Regulation of exogenous retroviruses and endogenous retroelements by MOV10

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The work presented in this thesis is my own

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ABSTRACT

Retroelements are some of the most successful parasites studied because of their ability to reverse transcribe and permanently integrate into the host genome. Host cells have, therefore, evolved multiple control mechanisms, such as cellular restriction factors, to protect their genomes from the pathogenic and mutagenic effects of retroelements. Identification of the full complement of these proteins is vital to comprehend the capacity of the host to regulate these genetic parasites. Human MOV10 is a putative RNA helicase with inhibitory or stimulatory roles in the replication of several RNA viruses, and the homologs of which play vital roles in the restriction of viruses and endogenous retroelements. Furthermore, MOV10 interacts with antiviral APOBEC3 proteins and core post-transcriptional RNA silencing machinery, all of which co-localise in cytoplasmic mRNA processing bodies and stress granules. Considering MOV10 cellular associations and homolog functions, the capacity of MOV10 to regulate the replication of a diverse panel of genetically distinct retroelements was investigated here.

Ectopically overexpressed MOV10 potently restricts the replication of retroviruses as well as the propagation of LTR and non-LTR endogenous retroelements. Significantly, RNAi-mediated silencing of endogenous MOV10 enhances the replication of endogenous retroelements, but not exogenous retroviruses demonstrating that natural levels of MOV10 suppress retrotransposition. MOV10 overexpression decreases the level of HIV-1 genomic RNA packaged into nascent virions and also impacts the accumulation of reverse transcription products in target cells. The molecular mechanism/s by which MOV10 inhibits retroelements remains unclear, however, the anti-retroelement activities of MOV10 and APOBEC3 proteins are independent. Moreover, MOV10 is not essential for miRNA-mediated translation repression or slicer activity in cultured cells. In sum, ectopically overexpressed human MOV10 inhibits divergent exogenous and endogenous retroelements and, more significantly, the capacity of endogenous MOV10 to specifically suppress retrotransposition highlights it as a potential restriction factor of human retrotransposons in somatic cells.

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ABBREVIATIONS

AGO	Argonaute
AGS	Aicardi-Goutières syndrome
AIDS	Acquired immunodeficiency syndrome
A-MLV	Amphotropic-MLV
APOBEC3	Apolipoprotein B mRNA editing enzyme polypeptide like 3
β-gal	β-galactosidase
CA	Capsid
CDA	Cytidine deaminase
CDK9	Cyclin-dependent kinase 9
CHMP	Charged multivesicular body protein
CMV	Cytomegalovirus
CPPT	Central polypurine tract
CRL4	Cullin4-RING ubiquitin ligase
CRM1	Chromosome region maintenance 1
C-terminal	Carboxy-terminal
CTL	Cytotoxic T lymphocyte
CypA	Cyclophilin A
DAPI	4',6-diamidino-2-phenylindole
DCAF1	DDB1-CUL4 associated factor 1
DCs	Dendritic cells
DDX3	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked
DDX6	DEAD (Asp-Glu-Ala-Asp) box helicase 6
dNTP	Deoxyribonucleotide triphosphates
eIF	Eukaryotic translation initiation factor
eIF4E-T	Eukaryotic translation initiation factor E transporter
E-MLV	Ecotropic-MLV
EN	Endonuclease
Endo-siRNA	Endogenous siRNA
Env	Envelope
ER	Endoplasmic reticulum
ERV	Endogenous retrovirus
ESCRT	Endosomal sorting complexes required for transport
FACS	Fluorescence activated cell sorting
G3BP1	RasGAP SH3-domain binding protein 1
Gag	Group specific antigen
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAART	Highly active antiretroviral therapy
HCV	Hepatitis C virus
HIV-1	Human immunodeficiency virus type-1
HIV-2	Human immunodeficiency virus type-2
HTLV-1	Human T-cell leukemia virus type-1
IAP	Intracisternal A-type particle
IFNα	Type I interferon α
IL-2	Interleukin-2
IL-4	Interleukin-4

IN	Integrase
IRES	Internal ribosome entry sequence
ISG	IFN-stimulatory gene
KD	Knockdown
LINE-1	Long interspersed nucleotide element-1
LTR	Long-terminal repeat
Luc	Luciferase
MA	Matrix
MDDCs	Monocyte-derived dendritic cells
MDMs	Monocyte-derived macrophages
MHC	Major histocompatibility complex
miRNA	Micro RNA
MLV	Murine leukemia virus
MMTV	Mouse mammary tumour virus
MOV10	Moloney leukemia virus 10
MOV10L1	MOV10-like 1
M-PMV	Mason-Pfizer monkey virus
NC	Nucleocapsid
Nef	Negative factor
NK	Natural killer
N-terminal	Amino-terminal
ORF	Open-reading frame
PBMCs	Peripheral blood mononuclear cells
P body	Processing body
PBS	Primer-binding site
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
$PI(4,5)P_2$	Phosphatidyl inositol (4,5) biphosphate
PIC	Pre-integration complex
piRNA	Piwi-interacting RNA
Pol	Polymerase
PPT	Polypurine tract
PR	Protease (processed enzyme)
Pro	Protease
Psi	Packaging signal
PTGS	Post-transcriptional gene silencing
qPCR	Quantitative PCR
R	Repeated
RBP	RNA-binding protein
RdRP	RNA-dependent RNA polymerase
Rev	Regulator of virion expression
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNA pol II	DNA-dependent RNA polymerase II
RNP	Ribonucleoprotein
RRE	Rev-response element
RRM	RNA recognition motif
RT	Reverse transcriptase
RTC	Reverse transcription complex

SAMHD1	Sterile α motif and HD domain-containing protein 1
SG	Stress granule
shRNA	Short hairpin RNA
SINE	Short interspersed nucleotide element
siRNA	Short-interfering RNA
SIV	Simian immunodeficiency virus
SIVcpz	Chimpanzee derived SIV
SIVmac	Rhesus macaque derived SIV
SIVsmm	Sooty mangabey monkey derived SIV
SV40	Simian vacuolating virus 40
TAR	Transactivation response
Tat	Transactivator of transcription
TCR	T cell receptor
TGS	Transcriptional gene silencing
TIA-1	T-cell restricted intracellular antigen 1
TPRT	Target-site primed reverse transcription
TREX1	3' exonuclease three prime repair exonuclease 1
TRIM5α	Tripartite motif-containing protein 5α
tRNA	Transfer RNA
U3	Unique 3'
U5	Unique 5'
UTR	Untranslated region
Vif	Virion infectivity factor
VLP	Virus-like particle
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
VSV	Vesicular stomatitis virus
YB-1	Y-box-binding protein 1

CHAPTER 1
INTRODUCTION

1.1 Retroviruses

The *Retroviridae*, or retrovirus family consists of a large group of divergent RNA viruses. Retroviruses replicate by reverse transcribing their single-stranded RNA genomes into copies of double-stranded DNA that are permanently integrated into the host chromosomal DNA. The integrated provirus serves as a template for transcription of the viral RNA, which is incorporated into virions or forms the mRNA for translation of viral proteins, allowing the virus to maintain a persistent infection. Unique steps such as reverse transcription and integration define, and also distinguish, retroviruses from all other virus families.

1.1.1 Taxonomic classification

Originally, retroviruses were classified according to the morphology of the virion core, for example, its shape and position, as visualised by electron microscopy. The genera have since been expanded and in addition to core morphology are also based on the organisation of the viral genome and site of virus assembly. Such criteria have allowed grouping of the genera into simple and complex retroviruses (Table 1.1). Both simple and complex retroviruses encode genes for the group specific antigen (Gag), protease (Pro), polymerase (Pol) and envelope (Env) proteins, however, the latter harbour additional genes for accessory and regulatory proteins that play important roles in viral replication (Goff, 2001). This thesis will focus predominantly on the human immunodeficiency virus type-1 (HIV-1); however, particular studies also extend to incorporate human immunodeficiency virus type-2 (HIV-2), rhesus macaque derived simian immunodeficiency virus (SIVmac), murine leukemia virus (MLV) and Mason-Pfizer monkey virus (M-PMV) and, therefore, these will also be briefly discussed in this chapter.

Table 1.1. Taxonomic classification of the *Retroviridae* family.

Genus	Example	Virion morphology	Genome	Assembly site
Alpharetrovirus	Avian leukosis sarcoma virus (ALSV)	Concentric, spherical core	Simple	Plasma membrane
Betaretrovirus	Mason-Pfizer monkey virus (M-PMV) Mouse mammary tumour virus (MMTV)	Concentric, cylindrical core Eccentric, spherical core	Simple (Complex)	Cytoplasm
Gammaretrovirus	Murine leukemia virus (MLV)	Concentric, spherical core	Simple	Plasma membrane
Deltaretrovirus	Human T-cell leukemia virus type-1 (HTLV-1)	Concentric, spherical core	Complex	Plasma membrane
Epsilonretrovirus	Walleye dermal sarcoma virus	Concentric, spherical core	Complex	Plasma membrane
Lentivirus	Human immunodeficiency virus type-1 (HIV-1)	Concentric, cylindrical or conical core	Complex	Plasma membrane
Spumavirus	Human foamy virus	Concentric, uncondensed spherical core	Complex	Cytoplasm

1.2 Retroviral pathogenesis

Retroviral infections can cause diseases that are directly tumourigenic or more cytopathic resulting in cell death and the development of immunodeficiencies as well as neuropathologies.

1.2.1 Oncogenic retroviruses

Acute transforming retroviruses are efficient and rapid inducers of tumours. These viruses express oncogenes (*v-onc*) acquired following a recombination event between viral and proto-oncogenic host sequences (*c-onc*) that are subsequently transferred onto

the viral genome. Inappropriate expression of the cellular oncogene during infection results in the formation of aggressive tumours. These viruses are normally replication-defective due to the loss of a viral gene required for replication during the acquisition of a host gene and, therefore, rely on a helper replication-competent virus for transmission (Goff, 2001). The Rous sarcoma virus carries the *c-src* gene and was the first oncogenic virus to be identified, although this virus is replication competent and, therefore, an exception to the rule.

Unlike acute transforming viruses, slow leukemia viruses are less efficient and slower at inducing tumours. These viruses are replication-competent and do not express any oncogenes; instead they form tumours by a mechanism called proviral insertional mutagenesis that involves the integration of a provirus, at random, within or in close proximity to a cellular proto-oncogene causing it to be activated (Goff, 2001). Several MLVs are slow leukemia viruses that induce the formation of thymic lymphomas.

1.2.2 Cytopathic retroviruses

Several retroviruses are the etiological agents for immunosuppressive diseases. Primate lentiviruses are the prototype associated with a chronic progressive disease and immunodeficiency in both human and non-human primates.

1.2.2.1 Origin of HIV

HIV-1 and HIV-2 are distantly related human lentiviruses that were identified as the causative agents of acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Clavel et al., 1986). HIV-1 consists of four subtypes: groups M (major), N (non-M, non-O), O (outlier) and P (pending the identification of further human cases). The group M subtype has infected people worldwide and is the cause of the HIV-1 pandemic. The remaining subtypes are extremely rare and restricted to parts of West and Central Africa. Similarly, HIV-2 is mainly restricted to West Africa and is normally associated with lower viral loads and transmission rates in comparison with HIV-1. Although the clinical symptoms of HIV-2 infections are similar to HIV-1, in most cases, HIV-2 infected individuals do not progress to AIDS (Sharp and Hahn, 2011).

The simian immunodeficiency viruses (SIVs) are lentiviruses that infect African non-human primates. HIV-1 and HIV-2 are a result of zoonotic transfers of chimpanzee derived SIV (SIVcpz) and sooty mangabey monkey derived SIV (SIVsmm), respectively, into humans (Gao et al., 1992; Gao et al., 1999)[reviewed in (Hahn et al., 2000)]. Likewise, SIVmac was identified not to be a natural pathogen of macaques, but a result of cross-species transmission of SIVsmm from mangabey monkeys in primate centres (Apetrei et al., 2005).

1.2.2.2 HIV and SIV pathogenesis

Both HIVs and SIVs infect target cells using CD4 as the primary cell surface receptor. In addition to T lymphocytes, CD4 is expressed on a range of cell types such as monocytes, macrophages and dendritic cells (DCs). Broadly, HIV and pathogenic SIV infections result in the profound depletion of CD4+ T cells and, therefore, impairment of their function resulting in the onset of immunodeficiencies, opportunistic infections and ultimately AIDS. Importantly, with the possible exception of SIVcpz, the majority of SIVs fail to cause disease in their natural African hosts (Keele et al., 2009).

Transmitted HIV-1 requires CD4 and the CCR5 co-receptor for infection (R5-tropic virus), consistent with the knowledge that mucosal CD4+ T cells are the initial targets for infection. CD4+ memory T cell subsets express high levels of CCR5 and are, therefore, the predominant cell type infected in both HIV and SIV infection (Douek et al., 2002). The virus and virus-infected cells at the site of exposure spread to proximal lymph nodes to expand from a localised infection to systemic infection. Furthermore, DCs can exacerbate infection by capturing and transmitting virus to T cells, although they are not productively infected (Lackner et al., 2011; Swanstrom and Coffin, 2011).

Acute infection is characterised by the availability of a large number of activated CD4+ T cells and high levels of viremia. This is ultimately controlled by the onset of early immune responses and, principally, a CD8+ cytotoxic T lymphocyte (CTL) response against infected cells (Swanstrom and Coffin, 2011). The massive population of CD4+ memory T cells in the gut-associated lymphoid tissue (GALT) is depleted more rapidly than those in other lymphoid tissues or the blood, and associated damage to the GALT and intestinal lining moves bacterial products into the blood activating a more generalised immune response. Interestingly, the activation of such a proinflammatory

immune response distinguishes pathogenic and non-pathogenic models of SIV infection [reviewed in (Brenchley and Douek, 2008)].

The onset of early immunodeficiency allows the virus to evolve and alter its tropism by using the CXCR4 co-receptor (X4-tropic virus), which correlates with rapid progression towards a disease state (Doranz et al., 1996; Koot et al., 1999). Naïve T cells express high levels of the CXCR4 co-receptor allowing X4-tropic viruses to infect these cells, and continually infect memory T cell subsets due to the expression of high levels of CXCR4 on these as well. Furthermore, late-evolving macrophage-tropic viruses can infect macrophages that express low levels of CD4 (Swanstrom and Coffin, 2011).

Immune activation increases the turnover of CD4+ memory T cells, therefore, moderately replenishing the 'stock' of available target cells. As HIV-1 replicates in activated CD4+ cells, chronic infection is characterised by a constant increase in the levels of viremia and, consequently, a gradual decrease in CD4+ T cell numbers. Ultimately the drop in CD4+ T cells reaches levels at which immune function is compromised causing opportunistic infections and the AIDS phase (Swanstrom and Coffin, 2011). The mechanism of CD4+ T cell death is not fully understood; however, it is clear that over the course of infection cell death is a result of multiple factors involving the direct killing of infected cells, indirect killing of uninfected bystander cells and chronic immune activation (Li et al., 2005; Doitsh et al., 2010; Lackner et al., 2011; Swanstrom and Coffin, 2011).

1.2.2.3 HIV-1 therapy

Currently, there is no cure for HIV-1 infection; however, to date, more than twenty Food and Drug Administration (FDA)-approved antiretroviral agents (ARVs) exist for the treatment of HIV-1. With the advent of combined therapy, which is also known as highly active antiretroviral therapy (HAART), viral loads can be reduced to below the limit of detection. The principle of HAART therapy is the use of three antiretroviral drugs that can target at least two separate molecular events in the viral life cycle preventing the evolution of drug resistant viral strains, which are common due to the highly error-prone reverse transcription process. The stages of the HIV-1 life cycle targeted by inhibitors so far are entry, reverse transcription, integration and protease processing. However, this list is by no means exhaustive and inhibitors of any stage of

the viral life cycle that are distinct from cellular functions may be potential targets. Furthermore, targeting of host cell restriction factors and cofactors may also provide a novel approach for treatment of HIV-1 infection (Arts and Hazuda, 2011).

During acute infection a latent reservoir of HIV-1 infected resting CD4+ memory T cells is established. A shortcoming of ARV therapy is its inability to clear latent infection, as these proviral integrations are not transcriptionally active and, therefore, do not produce viral proteins. Furthermore, the development of severe drug toxicities, drug resistant mutant strains and general failure of treatment due to factors such as poor tolerability and low CD4+ T cells counts when treatment is started illustrate that novel therapies are still required.

The development of a protective vaccine is an active area of research, the major obstacle of which is the heterogeneity between virus strains worldwide. A successful vaccine would require the induction of broadly acting and cross-reactive neutralising antibodies against the Env glycoprotein, and recent HIV and SIV vaccine trials have provided clues that will likely advance this field of vaccine development in the right direction (Rerks-Ngarm et al., 2009; Barouch et al., 2012). Vaccine research has also focused on the development of T-cell based vaccines that prevent the progression of disease, and primarily vaccines that can stimulate CD8+ CTL responses (Buchbinder et al., 2008).

1.2.2.4 M-PMV pathogenesis

M-PMV, also known as simian retrovirus type 3 (SRV-3) is a simian retrovirus that can lead to the development of a disease analogous to AIDS caused by pathogenic SIVs. Infection results in peripheral blood cytopenias causing immunodeficiency, ultimately resulting in the development of a range of pathological disorders attributed to opportunistic pathogen infections. The molecular mechanisms of pathogenesis, however, are not well understood [reviewed in (Montiel, 2010)].

1.3 Organisation of the retroviral genome

Retroviruses are 7 to 13kb single-stranded, positive sense RNA viruses that package two identical copies of their genome into nascent virions. The viral genome exists as a homodimer formed by interactions between the dimer linkage structures (DLS) found at the 5' ends of each RNA. As viral RNA is transcribed by the host transcriptional machinery it is capped and polyadenylated similar to cellular mRNAs. Several important *cis*-acting elements exist in the retroviral RNA (Figure 1.1A); the repeated (R) region is present at both termini, which is followed by the unique 5' sequence (U5) at the 5' end and preceded by the unique 3' sequence (U3) at the 3' end. The primer-binding site (PBS) is located immediately downstream of the U5 region and is required for the initiation of reverse transcription (section 1.5.3). The PBS is followed by the viral RNA packaging signal (Psi). The polypurine tract (PPT), which also plays an important role in later stages of reverse transcription, is located immediately upstream of the U3 region (Goff, 2001). Many cis-acting elements are unique to lentiviruses, such as HIV-1 (Figure 1.1A, illustrated in red). In addition to the PPT at the 3'end of the viral RNA these also include a central polypurine tract (cPPT) (Charneau et al., 1992). Additionally, these viral RNAs contain two complex structures: the transactivation response (TAR) element and the Rev-response element (RRE) that are involved in transcription of the viral RNA and nuclear export, respectively (Malim et al., 1989; Dingwall et al., 1990) (sections 1.5.6 and 1.5.7).

Following reverse transcription and integration of the retroviral cDNA, the viral RNA is transcribed and translation of the viral proteins ensues. The sequences between the Psi and PPT are mainly coding regions for viral proteins, which for most simple retroviruses encode *gag*, *pro*, *pol* and *env*, and for the complex retroviruses, such as HIV-1 and HIV-2, encode additional genes for accessory and regulatory proteins (Figure 1.1B). All primate lentiviruses encode the regulatory proteins transactivator of transcription (Tat) and regulator of virion expression (Rev), and the accessory proteins virion infectivity factor (Vif), viral protein R (Vpr) and negative factor (Nef). Additionally HIV-1/SIVcpz also encode viral protein U (Vpu), whereas HIV-2/SIVsmm/SIVmac encode the viral protein X (Vpx) instead.

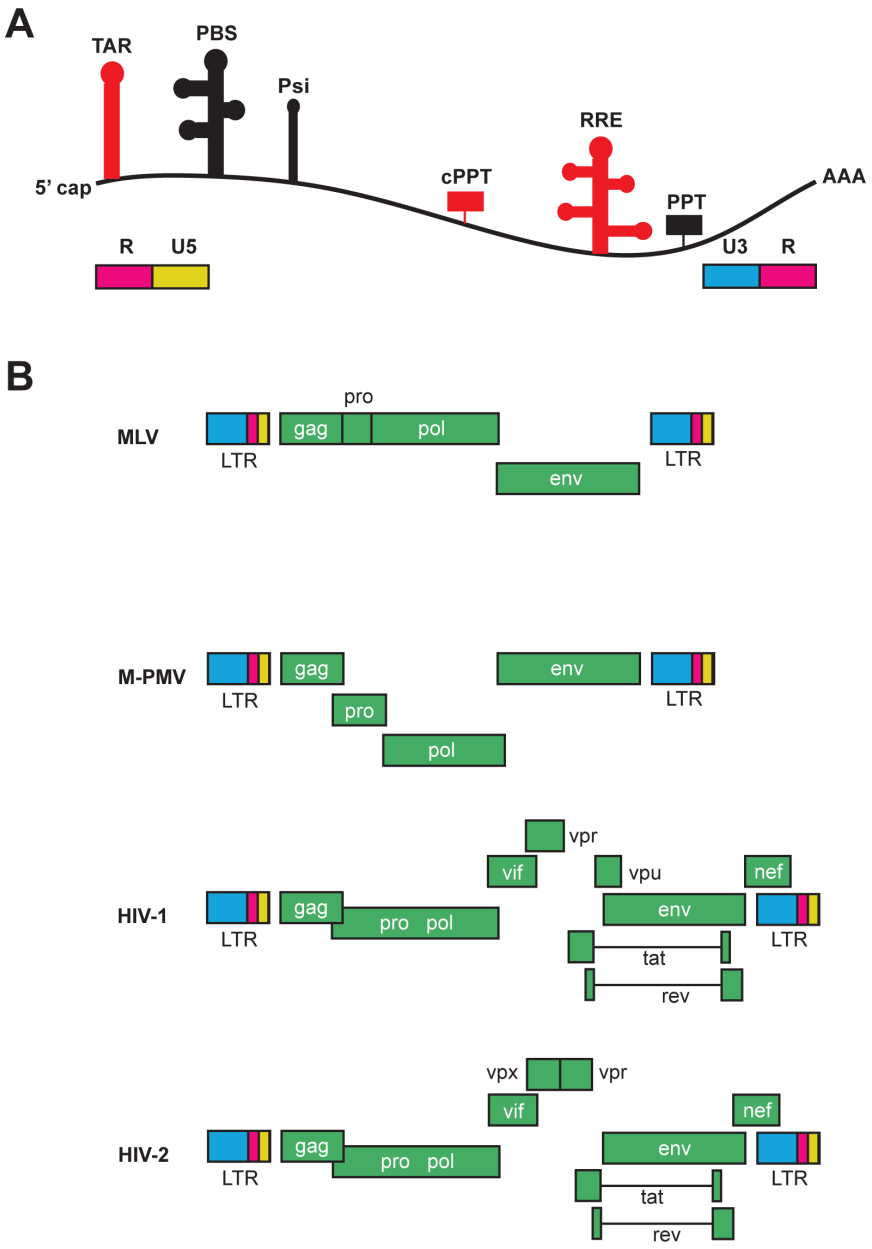


Figure 1.1. Organisation of the retroviral genome.

(A) Retroviral RNA *cis*-acting elements. All retroviral RNAs are capped at the 5' end, polyadenylated at the 3' end and contain a primer-binding site (PBS), packaging signal (Psi) and polypurine tract (PPT) (Illustrated in black). The repeated (R) regions, unique 5' (U5) and unique 3' (U3) sequences are also depicted. Lentiviruses contain additional cis-acting elements such as the transactivation response (TAR) element, central polypurine tract (cPPT) and Rev-response element (RRE) (Illustrated in red). Adapted from (Goff, 2001) and (Freed and Martin, 2001). **(B) Simple and complex retrovirus protein coding regions.** Both simple retroviruses (MLV and M-PMV) and complex retroviruses (HIV-1 and HIV-2) encode *gag*, *pro*, *pol* and *env,* and additionally HIV-1 and HIV-2 also encode *tat*, *rev*, *vif*, *vpr*, *vpu* (HIV-1 only), *vpx* (HIV-2 only) and *nef.* Adapted from (Goff, 2001) and (Freed and Martin, 2001).

1.4 Mature retroviral virion

Virions are released from infected cells as immature particles containing partially processed Gag and Gag-Pro-Pol proteins. These are eventually fully processed into mature infectious particles that are roughly 100nm in diameter, although this can vary. Processing of Gag and Gag-Pro-Pol precursors results in the production of the Gag proteins nucleocapsid (NC), capsid (CA), matrix (MA) and other Gag products specific to the virus (Figure 1.2A). Pro is processed into the enzyme protease (PR), and Pol is cleaved to produce the reverse transcriptase (RT) and integrase (IN) enzymes. The viral genomic RNA is associated with NC in the virion, which is enclosed within a core made up of the CA protein. The shape of the virion core can vary depending on the virus (section 1.1.1). The MA protein loosely forms a shell around the virion core, and all the virion components are enclosed within a lipid bilayer containing the Env glycoprotein (Goff, 2001) (Figure 1.2B). Cellular enzymes process the Env precursor into gp41 (transmembrane subunit) and gp120 (surface unit subunit), the latter of which is an extravirion component observed as protrusions from the virion membrane. Cellular proteins are also packaged into virions, some of which will be discussed in more detail in later sections.

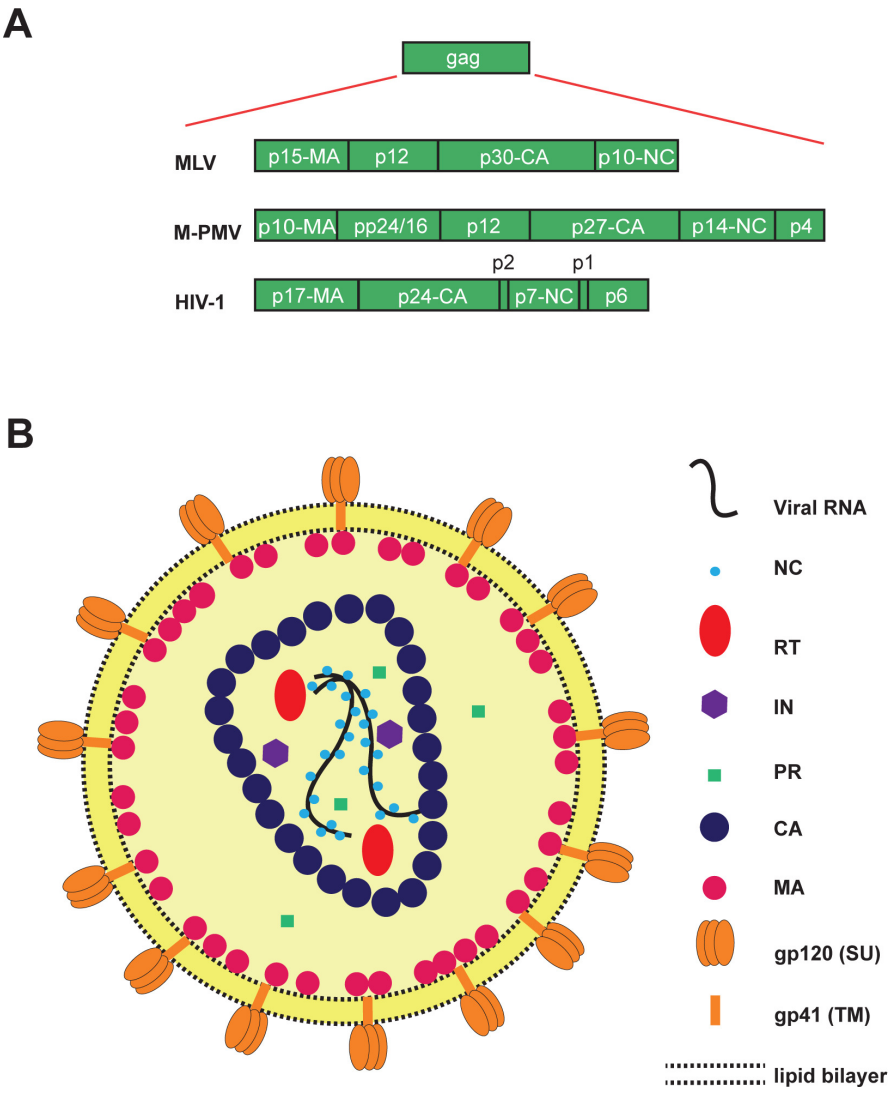


Figure 1.2. Mature retroviral virion.

(A) Processed Gag domains. Precursor retroviral Gag proteins are processed into matrix (MA), capsid (CA), nucleocapsid (NC) and additional domains dependent on the virus, for example, the spacer peptides p2, p1 and the p6 domain in the case of HIV-1. **(B) Organisation and components of the mature retroviral virion.** The mature virion consists of a dimeric RNA genome, the structural Gag proteins NC, CA and MA, the viral enzymes reverse transcriptase (RT), integrase (IN) and protease (PR), encompassed in a lipid bilayer containing trimeric complexes of the Env protein gp41 and gp120 domains. Adapted from (Goff, 2001).

1.5 Retroviral life cycle

The retroviral life cycle is initiated with an RNA genome that traverses through a DNA intermediate and returns again to RNA form for packaging into virions. These basic requirements of viral replication are accomplished via a number of specific and intricate viral processes (Figure 1.3). The early phase of the retroviral life cycle is initiated with entry of the virus particle into a target cell by interacting with a specific cell surface receptor through the Env protein. Receptor binding induces fusion of the viral and cellular membranes and subsequent internalisation of the viral core into the cell cytoplasm (section 1.5.1). Uncoating of the viral capsid may occur prior to or during and following reverse transcription depending on the virus (section 1.5.2). The process of reverse transcription is mediated by the RT enzyme, which catalyses the conversion of the viral RNA genome into a DNA copy that is imported into the nucleus and integrated into the host chromosomal DNA by the IN enzyme (sections 1.5.3 to 1.5.5). The late phase of the viral life cycle commences with transcription of the viral RNA coupled to elaborate splicing events and nuclear export of both unspliced and spliced viral transcripts into the cytoplasm (sections 1.5.6 and 1.5.7). Viral proteins are translated and traffick either to specific sites in the cytoplasm or the plasma membrane for assembly and packaging of the viral RNA (sections 1.5.8 and 1.5.9), following which immature virus particles bud through the plasma membrane (section 1.5.10). The PR enzyme cleaves and processes the viral structural and enzymatic proteins generating a mature virion that is able to infect a new target cell and repeat the cycle (sections 1.4 and 1.5.11). Due to the limited size of the retroviral genome an array of host proteins, or cofactors, are also hijacked by the virus to perform important functions in the viral life cycle. These will be discussed in more detail in relevant sections of this chapter.

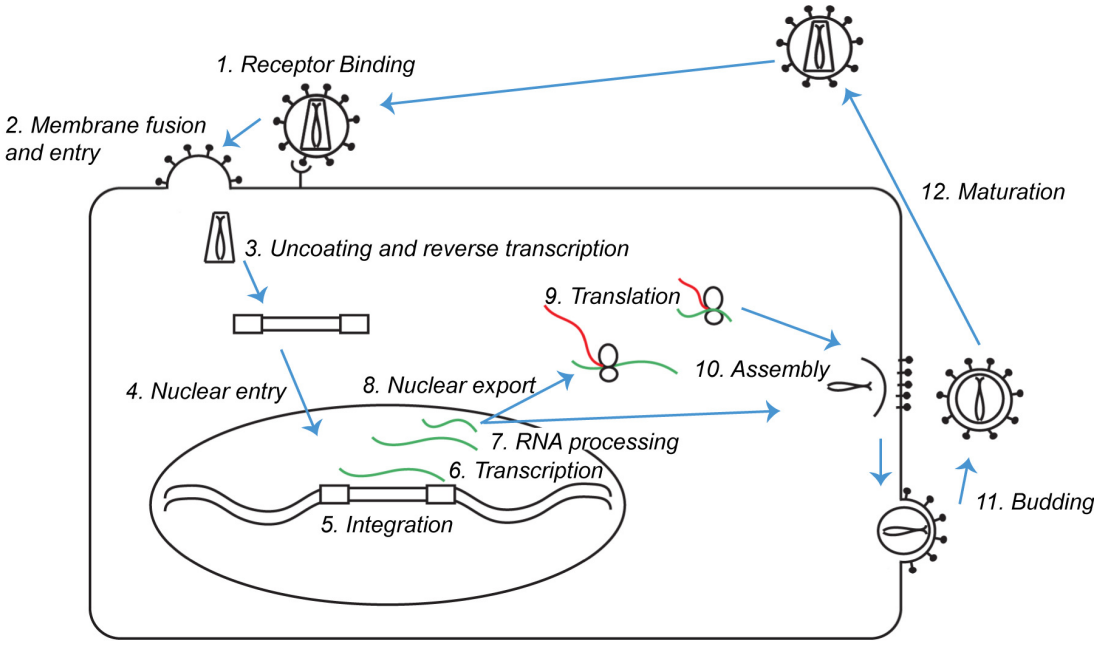


Figure 1.3. Retroviral life cycle.

Mature retroviral particles bind to a specific cellular surface receptor via the Env glycoprotein (1) allowing fusion and entry of the viral core into the cell cytoplasm (2). Following uncoating of the viral capsid and reverse transcription of the single-stranded RNA genome (3) the double-stranded DNA product enters the nucleus of the cell (4) and is integrated into the host chromosomal DNA (5). Transcription (6) and processing of the viral transcripts (7) ensues, following which viral mRNAs are exported into the cytoplasm of the cell (8) for translation of the viral proteins (9) and assembly of nascent virions (10). Immature virions bud through the cell plasma membrane (11) and are processed into mature virions (12) that are able to infect new target cells and repeat the cycle. Adapted from (Goff, 2001).

1.5.1 Entry

Retroviral entry is a complex, multi-step process initiated by binding of the viral Env glycoprotein to a specific cell surface receptor. A diverse range of receptors are utilised for entry and these determine the cell type specificity of the virus, or viral tropism (Table 1.2) (Goff, 2001). Subsequently, the viral Env protein undergoes radical conformational changes to expose the fusion peptide allowing fusion of the viral and cellular lipid bilayers. Fusion is pH-independent for the majority of retroviruses, including HIV-1, suggesting that changes in the conformation of Env are not dependent on an endosomal acidification step and likely occur at the plasma membrane (Stein et al., 1987), although alternative theories have been proposed (Miyauchi et al., 2009). The viral core is internalised and delivered to the cytoplasm following the membrane fusion event.

Table 1.2. Retroviral receptors.

Genus	Example	Receptor	Function
Betaretrovirus	M-PMV	RDR	Neutral amino acid transporter
Gammaretrovirus	Ecotropic-MLV (E-MLV)	mCAT-1	Cationic basic amino acid transporter
	Amphotropic-MLV (A-MLV)	rPiT-2	Phosphate transporter
	Xenotropic-MLV (X-MLV) & Polytropic-MLV (P-MLV)	XPR1	G-protein coupled signalling and/or phosphate transporter
Lentivirus	HIV and SIV	CD4 and CCR5 or CXCR4	Cellular immune response and G-protein coupled chemokine receptors

M-PMV (Tailor et al., 1999), E-MLV (Kim et al., 1991), A-MLV (Kavanaugh et al., 1994), X-MLV & P-MLV (Battini et al., 1999; Yang et al., 1999)

CD4 was the first retroviral entry receptor identified and is used by the majority of primate as well as feline lentiviruses (Maddon et al., 1986; McDougal et al., 1986). This receptor is a member of the immunoglobulin superfamily that functions in immune recognition and T cell receptor (TCR) signalling. CD4 usage explains the narrow tropism of viruses such as HIV-1, which is mainly restricted to T lymphocytes and macrophages (section 1.2.2.2). Interestingly, studies illustrated that CD4 expression alone was not sufficient for HIV-1 fusion (Maddon et al., 1986), which led to the identification of the chemokine receptors CCR5 and CXCR4 that serve as virus coreceptors (Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). Individuals that are homozygous for the CCR5Δ32 allele are largely resistant to HIV-1 infection, providing evidence for the importance of this coreceptor (Liu et al., 1996; Samson et al., 1996).

For HIV and SIV entry, gp120 binds to CD4 causing rearrangements in gp120 that allow engagement with the CCR5 or CXCR4 coreceptors. Viruses that utilise the CCR5 coreceptor are termed R5-tropic and those that use the CXCR4 coreceptor are called X4-tropic viruses (section 1.2.2.2). R5X4-tropic viruses also exist that can utilise both coreceptors. Evidence suggests that following receptor binding, viruses utilise host cell machinery, such as the actin cytoskeleton, to arrive at sites for membrane fusion by a mechanism termed 'surfing' (Lehmann et al., 2005). Coreceptor binding exposes the gp41 fusion peptide, which is inserted into the host plasma membrane resulting in the formation of a six-helix bundle that brings the viral and cellular membranes in close proximity. Formation of a fusion pore allows delivery of the viral core into the cell cytoplasm (Wilen et al., 2011).

1.5.2 Uncoating

Uncoating refers to disassembly of the viral core. Retroviruses such as the primate lentiviruses can replicate in terminally differentiated, non-dividing cells as they actively import their viral genome through nuclear pores into the nucleus (Lewis et al., 1992). Alternatively, gammaretroviruses such as MLV can only replicate in dividing cells as they access the nuclear chromatin following breakdown of the nuclear membrane during mitosis (Roe et al., 1993). The viral uncoating process may be at least partially responsible for these differences in mechanism of nuclear import and ability to infect

dividing and non-dividing cells. The diameter of the retroviral core exceeds that of the nuclear pore; therefore, early uncoating may be necessary for active nuclear import [reviewed in (Fassati, 2006)]. Evidence suggests that HIV-1 uncoating occurs early in infection based on the detection of very small amounts of viral CA associated with intracellular reverse transcription and pre-integration complexes (RTC and PIC) (Farnet and Haseltine, 1991; Fassati and Goff, 2001). On the other hand, the analysis of RTCs from MLV infection indicates that uncoating is a more gradual process occurring during reverse transcription and nuclear entry (Fassati and Goff, 1999). However, the timing of HIV-1 uncoating is somewhat controversial, whereby a particular model suggests that viral core disassembly takes place at the nuclear pore upon completion of reverse transcription (Iordanskiy et al., 2006).

The mechanism of uncoating is not very well understood, however, a mature virion is necessary for efficient uncoating of the viral capsid. Furthermore, the host cell factor cyclophilin A (CypA), which has been shown to be necessary for efficient HIV-1 infection, may function by facilitating uncoating (Braaten et al., 1996; Ylinen et al., 2009). More recently, the HIV-1 IN enzyme has also been implicated in the process of uncoating (Briones et al., 2010). On the contrary, host cell restriction factors tripartite motif-containing protein 5α (TRIM5α), TRIMCyp and Fv1 inhibit retroviral infection by affecting the uncoating step and, therefore, downstream processes such as reverse transcription or nuclear entry (sections 1.8.2.2 and 1.8.2.3).

1.5.3 Reverse transcription

Reverse transcription is one of the defining hallmarks of the retrovirus life cycle, which involves copying of the single-stranded RNA genome to double-stranded DNA for integration into the host genome. Although uncoating of the viral capsid is not fully understood, it is thought that exposure of the viral RNA to high levels of deoxyribonucleotide triphosphates (dNTPs) in the cytoplasm is sufficient for the initiation of reverse transcription. The majority of this process takes place in target cells, however, very low amounts of early DNA products can be detected in virion preparations (Trono, 1992). Exceptions to this rule are spumaviruses, which reverse transcribe predominantly during virus assembly so that the main form of genome in the virion is DNA (Goff, 2001).

The RT enzyme mediates reverse transcription and contains both DNA polymerase activity, incorporating dNTPs on a template, and RNase H activity, degrading RNA in a duplex (Baltimore, 1970; Mizutani et al., 1970; Molling et al., 1971). Different retroviral PR enzymes cleave Pol to produce RT enzymes with varying subunit structures dependent on the retrovirus. For example, the MLV RT enzyme is a monomer in solution, whereas the HIV-1 RT enzyme is a heterodimer consisting of a larger subunit with both DNA polymerase and RNase H activity (p66) and a smaller domain with only DNA polymerase activity (p51) (Goff, 2001). Reverse transcription occurs as part of a RTC containing multiple host and viral proteins. For HIV-1, the presence of CA in the RTC is controversial, however, other viral proteins such as MA, NC, IN and Vpr have been detected (Fassati and Goff, 2001; Nermut and Fassati, 2003; Iordanskiy et al., 2006).

The process of reverse transcription involves a series of detailed steps (Figure 1.4), initiated with synthesis of the minus strand strong stop DNA; the 3' end of a specific cellular transfer RNA (tRNA) primer, which is dependent on the virus, anneals to the complementary PBS in the plus strand RNA genome. HIV-1 uses the $tRNA^{Lys3}$. The RT enzyme synthesises this early DNA product towards the 5' end of the viral RNA using it as a template, producing the U5 and R sequences. The RNA component of the RNA: DNA hybrid formed during this step is degraded by the RT enzyme RNase H activity. This degradation allows transfer of the minus strand strong stop DNA from the 5'end of the viral genome to the 3'end, where it anneals to the complementary R region. This process is termed minus strand transfer. Using the minus strand strong stop DNA as a primer, minus strand DNA synthesis extends towards the 5' end of the viral genome till the PBS. Again, formation of an RNA: DNA hybrid results in degradation of viral RNA by RNase H activity, although the PPT is particularly resistant to degradation and remains annealed to the minus strand DNA. The short PPT RNA sequence serves as a primer for the initiation of plus strand strong stop DNA synthesis and elongates towards the 5' end of the minus strand DNA template generating the U3, R and U5 sequences as well as some of the complementary tRNA primer sequence. The PPT RNA sequence is degraded following this step and the tRNA primer at the 5'end of the minus strand DNA is also degraded by RNase H activity. Exposure of the 3' end of the plus strand strong stop DNA results in a second translocation step termed plus strand transfer, during which the plus strand strong stop DNA jumps to the 3'end

of the minus strand DNA and anneals with the PBS. The process of reverse transcription is completed with elongation of the 3' ends of both the minus strand DNA and plus strand DNA, ultimately resulting in the formation of a linear double-stranded DNA product that is the substrate for integration into the host genome. The U3-R-U5 blocks at either end of the DNA, referred to as the long-terminal repeats (LTRs), have important roles in the integration process (Goff, 2001; Hu and Hughes, 2011).

In addition to the PPT at the 3' end, lentiviruses such as HIV-1 have an additional cPPT located near the middle of the viral genome (section 1.3). This results in the initiation of a second plus strand synthesis event and the presence of a discontinuity in the DNA owing to termination of elongation from upstream primers. In the case of HIV-1, this creates a DNA overlap or flap that is thought to play an important role in replication (Charneau et al., 1992; Charneau et al., 1994).

HIV-1 sequences vary considerably within the same individual due to the introduction of mutations into the viral genome during replication. The error-prone RT enzyme that lacks a proofreading function contributes significantly to this mutation rate. Matters are complicated further by the recombination rate of retroviruses, which is highest for HIV-1, whereby viral templates are switched during reverse transcription generating a chimeric DNA that contains sequences from both viral RNA copies; this process results in the generation of a recombinant virus only in the instance that both viral template sequences are not identical. Viral divergence allows the virus to evade the host immune response and also antiviral therapies (Hu and Hughes, 2011).

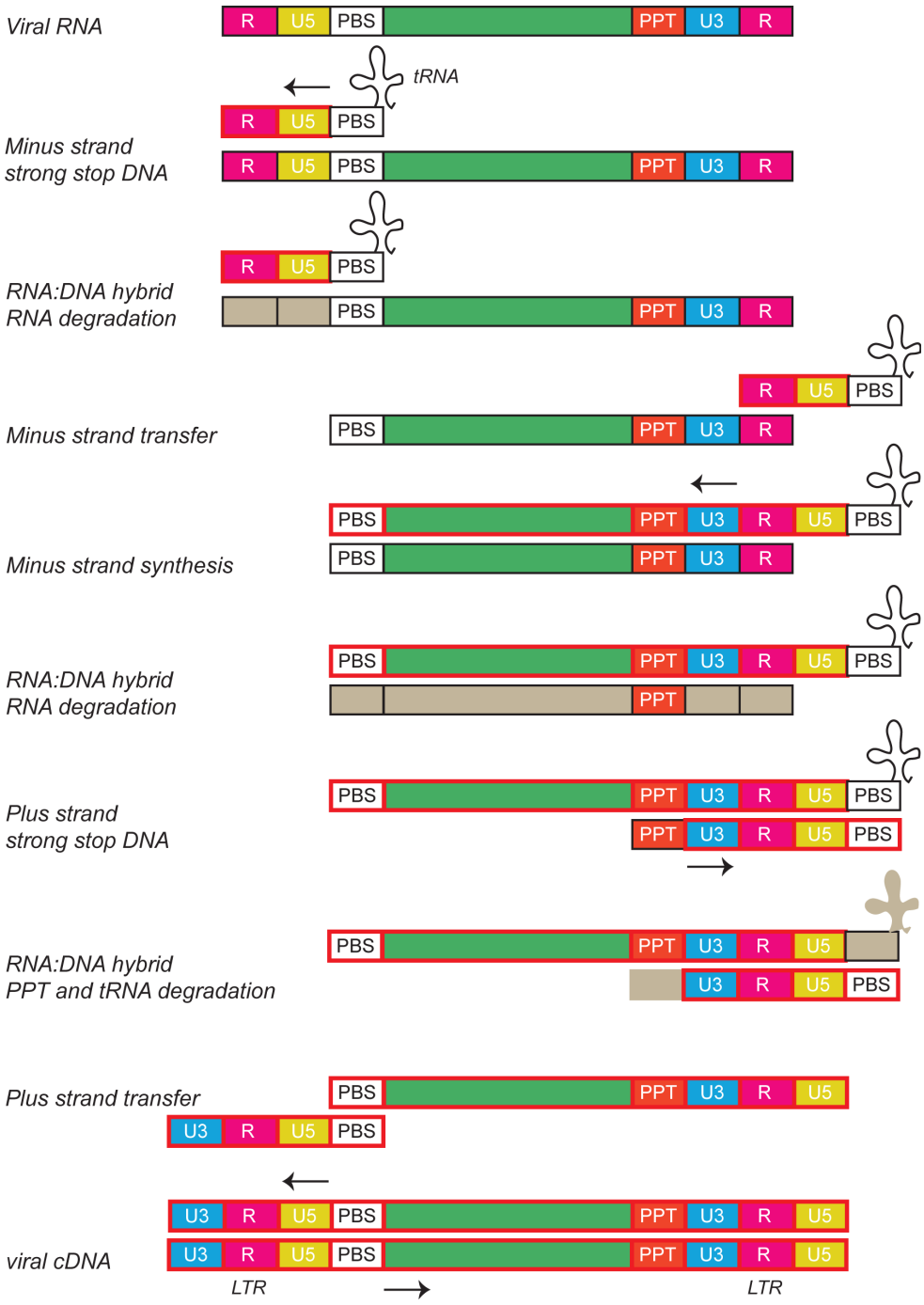


Figure 1.4. Reverse transcription.

A specific cellular tRNA primer anneals to the viral RNA PBS initiating the process of reverse transcription. The minus strand strong stop DNA is synthesised and the viral RNA component of the resultant RNA: DNA hybrid is degraded by RNase H activity. The minus strand strong stop DNA is transferred to the 3' end of the viral RNA and the minus strand DNA is synthesised. The viral RNA in the RNA: DNA hybrid is degraded by RNase H activity, leaving only the PPT RNA sequence intact. The remnant PPT sequence primes synthesis of the plus strand strong stop DNA. Both the PPT sequence and tRNA primer in the resultant RNA: DNA hybrid are degraded by RNase H activity. The plus strand strong stop DNA is transferred to the 3' end of the minus strand DNA template. Both the minus strand and plus strand DNA products are fully extended generating the final long-terminal repeat (LTR) containing viral cDNA product. DNA is depicted with a red border and RNA degradation by RNase H activity is depicted in grey. Adapted from (Goff, 2001) and (Hu and Hughes, 2011).

1.5.4 Nuclear entry

The viral cDNA must enter the nucleus for integration into the host chromosomal DNA, a process that is not well understood. It is thought that the RTC converts to the PIC as reverse transcription progresses and, therefore, many of the cellular and viral proteins found in the RTC also remain in the PIC. As discussed, the difference in ability of gammaretroviruses and lentiviruses to replicate in dividing and non-dividing cells is down to the mechanism of nuclear import, and viral core uncoating may influence the pathway adopted (section 1.5.2). Furthermore, viral and host proteins have also been implicated in actively importing the PIC into the nucleus.

The amino-terminal (N-terminal) region of the HIV-1 MA protein has been proposed to contain a nuclear localisation signal (NLS), the mutation of which was reported to block the ability of the virus to replicate in terminally differentiated cells (Bukrinsky et al., 1993); however, subsequent studies have not been able to confirm these findings (Fouchier et al., 1997). The HIV-1 accessory protein Vpr also contains at least two NLS domains and can bind directly to nucleoporins (Di Marzio et al., 1995; Fouchier et al., 1998), however, HIV-1 vector systems that lack Vpr are still able to infect non-dividing cells (Zufferey et al., 1997). The HIV-1 IN enzyme is karyophilic and putative NLS domains as well as an ability of IN to bind importins has been proposed (Gallay et al., 1997), although, the exact mechanism by which IN enters the nucleus is still a matter of debate (Devroe et al., 2003). Interestingly, HIV-1 viruses in which IN has been replaced with the MLV IN enzyme can still replicate in non-dividing cells (Yamashita and Emerman, 2005). Another HIV-1 element suggested to be required for nuclear import is the cPPT, however, spreading replication assays with wild-type virus containing a mutated cPPT only provide a modest inhibition of HIV-1 infection with no obvious defect in nuclear import (Limon et al., 2002).

A number of cellular factors have also been implicated in HIV-1 nuclear entry, such as importin7 and also tRNAs (Fassati et al., 2003; Zaitseva et al., 2006). Furthermore, genome-wide studies to identify cellular factors required for HIV-1 replication have identified a number of nuclear pore proteins to be required for nuclear entry (Lee et al., 2010; Zhang et al., 2010; Matreyek and Engelman, 2011; Schaller et al., 2011). Although transportin 3 was originally thought to be necessary for nuclear entry a post-nuclear entry role for transportin 3 has more recently been proposed (Christ et al., 2008;

Zhou et al., 2011). Further studies are required to identify the full range of factors and exact mechanisms involved in facilitating this viral process.

1.5.5 Integration

The second distinguishing step of the retroviral life cycle is integration. Following nuclear entry the viral cDNA is permanently integrated into the host genome, a step that is responsible for viral persistence, the tumourigenic properties of oncogenic viruses as well as vertical transmission of certain viruses into the germline. In the nucleus, the viral DNA can exist in linear double-stranded DNA form or as closed circles containing one copy of the LTR formed by homologous recombination between the LTRs, or two copies of the LTRs formed by blunt-end ligation. Non-functional closed circles are also formed by autointegration of DNA ends into internal sites within the viral DNA resulting in deletions and mutations. It is clear that the linear DNA form is the substrate for integration and that closed circles are dead-end products (Ellis and Bernstein, 1989; Lobel et al., 1989).

The IN enzyme, which is also processed from Pol by the PR enzyme, performs the integration step (Schwartzberg et al., 1984). The N-terminal region of IN contains a zinc-finger motif, the central region contains the catalytic activity and the carboxy-terminal (C-terminal) region is a less well-conserved DNA-binding domain (Engelman and Craigie, 1992). Similar to the RT enzyme, IN exists within the PIC in infected cells and does not function alone. Integration of the viral DNA takes place in two steps termed 3' end processing and strand transfer, and the *att* sites in the LTRs of the linear viral DNA, which are recognised by IN, are important for both of these processes (Figure 1.5). The first of these steps involves removal of two terminal nucleotides from the 3' ends of the linear DNA substrate by the IN enzyme. Cleavage occurs at a highly conserved *ca* sequence and in the case of most retroviruses releases a *tt* dinucleotide. During strand transfer, the generated free 3' OH ends of the viral DNA attack the phosphodiester bonds of the target chromosomal DNA forming a new phosphodiester bond between the viral DNA and the host DNA (Fujiwara and Mizuuchi, 1988). This process results in the formation of staggered ends at the site of attack that are repaired by the cellular machinery, possibly assisted by RT or IN, generating target site duplications at either end of the provirus. The end product is a clean insertion of the

viral DNA, consisting of the 5' LTR and 3' LTR flanking viral sequences, into the host chromosomal DNA (Goff, 2001; Craigie and Bushman, 2011).

Proviral integrations are generally random and can technically mutate any gene; however, studies have shown that retroviruses do have preferential aspects for target sites. For example, HIV-1 insertions predominate in highly active or transcribed regions, whereas, MLV inserts itself upstream of transcriptional start sites (Schroder et al., 2002; Wu et al., 2003). These differences in preference may be owed to the half-life of infected cells, or alternatively, the pathway of nuclear import. HIV-1 and MLV IN swap experiments resulted in the integration of chimeric HIV-1 near transcription start sites, analogous to MLV, implicating the IN enzyme in determining the target site for insertion amongst other factors (Lewinski et al., 2006). Interestingly, the cellular protein lens epithelium-derived growth factor (LEDGF/p75) is an essential cofactor for HIV-1 integration and target site selection (Llano et al., 2006; Shun et al., 2007). LEDGF/p75 interacts with IN and tethers it to the chromatin, and disruption of this interaction has been shown to inhibit HIV-1 infection (Maertens et al., 2003; Emiliani et al., 2005).

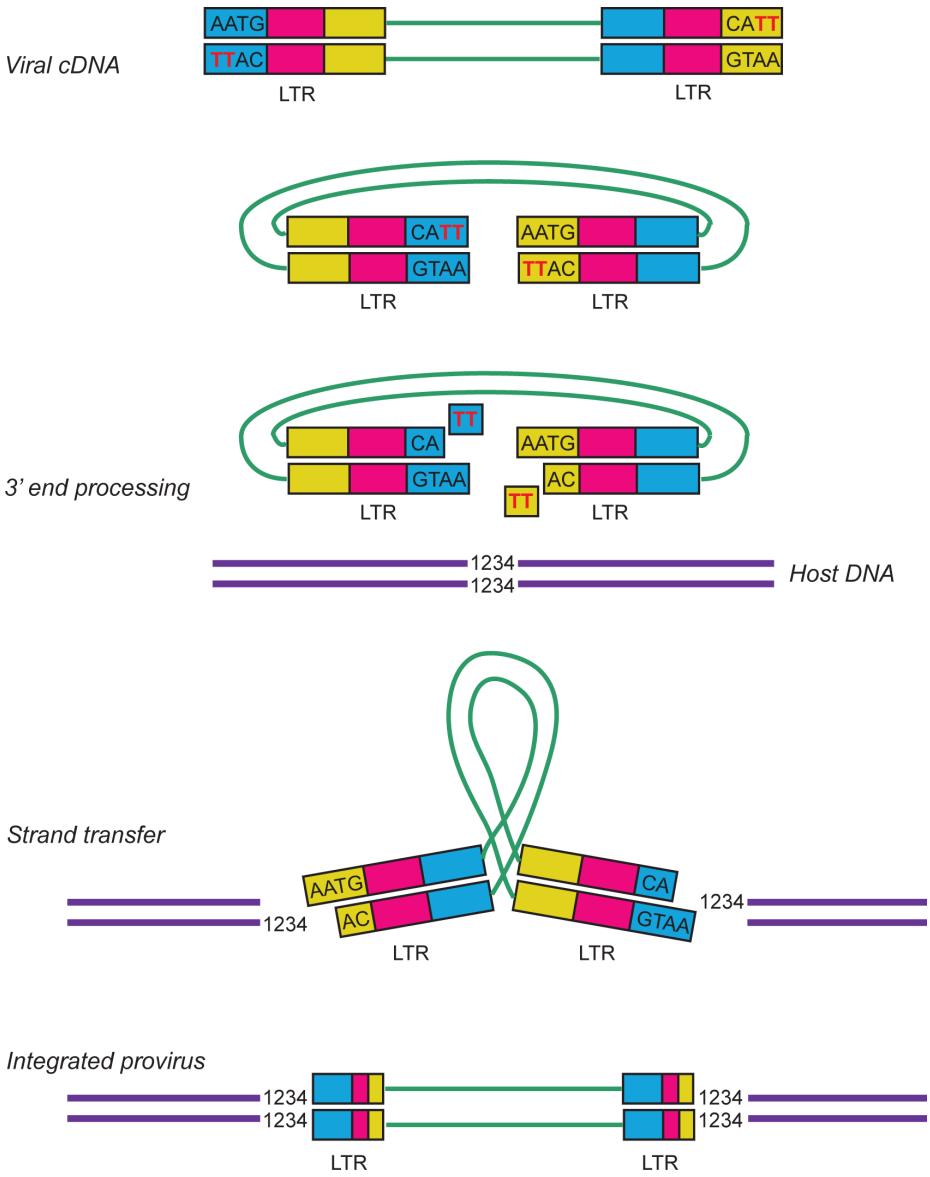


Figure 1.5. Integration.

The linear viral cDNA is integrated into the host chromosomal DNA through two processes termed 3' end processing and strand transfer. The IN enzyme recognises and processes *att* sites located at the 3' ends of the viral cDNA releasing two terminal nucleotides (generally a *tt* dinucleotide). The resultant 3' OH ends generate staggered breaks in the host DNA, following which new phosphodiester bonds form between the viral DNA and host DNA producing the integrated provirus. . Adapted from (Goff, 2001).

1.5.6 Transcription

Post-integration steps of the retroviral life cycle are often referred to as the late phase of viral replication, and in contrast to the early phase are regulated mainly by cellular proteins performing functions such as transcription and translation. Transcription of the viral RNA is mediated by the cellular DNA-dependent RNA polymerase II (RNA pol II). The viral promoter, consisting of core promoter elements as well as enhancer elements, is located in U3 and transcription is initiated at the U3-R border. The 5'end of the viral RNA is capped akin to cellular mRNAs by host capping machinery. Transcription continues through the 3' LTR and into the downstream flanking host DNA, following which the RNA is cleaved and polyadenylated at the R-U5 border due to the presence of a polyadenylation signal at the end of R. This long primary transcript is exported into the cytoplasm and either incorporated into assembling virions as the viral genome or forms the mRNA for translation of Gag and Gag-Pro-Pol precursor proteins; whether the same viral transcript can serve both functions is unclear, although a report has proposed that a single pool serves interchangeably for both functions (Butsch and Boris-Lawrie, 2000). Transcripts are also spliced into subgenomic mRNAs for the translation of other viral proteins, which for simple retroviruses is a single spliced mRNA encoding the Env protein, however, for complex retroviruses involves several alternatively spliced mRNAs for the translation of accessory and regulatory proteins (Goff, 2001).

In the case of HIV-1 infection, short fully spliced mRNAs encoding the regulatory proteins Tat and Rev are produced immediately after infection. Unusually for a transcription factor, Tat binds to an RNA hairpin structure called TAR in the R region that recruits cellular factors and enhances transcription elongation through the 5' LTR and into the viral genome. (Kao et al., 1987; Dingwall et al., 1990). The cellular positive transcription elongation factor (P-TEFb) complex consists of the protein Cyclin-T1 reported to bind to cyclin-dependent kinase 9 (CDK9), Tat and TAR, and is an important cofactor for Tat-mediated transactivation of viral RNA transcription (Mancebo et al., 1997; Bieniasz et al., 1998; Wei et al., 1998). Binding of Tat to the Cyclin-T1 component of P-TEFb induces conformational changes in the complex that result in CDK9-mediated phosphorylation of both positive and negative regulators of elongation. These include factors such as the negative elongation factor (NELF), the DRB sensitivity-inducing factor (DSIF) and importantly the C-terminal domain of RNA

pol II, facilitating the maintenance of Tat-mediated transcription elongation (Kim et al., 2002; Fujinaga et al., 2004; Yamada et al., 2006). Recent studies have also provided evidence for the recruitment of additional transcription and elongation factors by Tat, such as AF4/FMR2 family, member 4 (AFF4), eleven nineteen leukemia (ENL), ALL1-fused gene from chromosome 9 protein (AF9) and elongation factor, RNA polymerase II, 2 (ELL2) (He et al., 2010).

1.5.7 RNA processing and nuclear export

Viral transcripts are exported from the nucleus following transcription for subsequent assembly steps and the translation of viral proteins. Interestingly, intron-containing viral mRNAs are exported into the cytoplasm, despite the fact that cellular mRNAs are prevented from export until they are fully spliced. Retroviruses evade splicing in a number of ways, a general mechanism for which may be the inefficient recognition of viral splice sites by the cellular spliceosome (Katz and Skalka, 1990). Simple retroviruses such as M-PMV produce unspliced mRNAs that serve as the viral genome and encode *gag*, *pro* and *pol*. Alternatively, the Env protein is translated from a fully spliced transcript. Cis-acting elements termed the constitutive transport elements (CTEs) located at the 3' end of M-PMV unspliced and fully spliced mRNAs mediate nuclear export by interacting directly with the cellular NXT/NXF1 nuclear export pathway (Bray et al., 1994; Wiegand et al., 2002).

Complex retroviruses encode genes for regulatory and accessory proteins in addition to *gag*, *pro*, *pol* and *env*. The HIV-1 RNA contains four splice donors and eight splice acceptor sites resulting in the production of a multitude of partially spliced and fully spliced mRNAs (Figure 1.6). A 9kb unspliced primary transcript can be incorporated into virions as the viral genome, and also serves as the template for translation of structural and enzymatic Gag and Gag-Pro-Pol proteins. A bicistronic partially spliced 4kb mRNA encodes for both the Env protein and the accessory protein Vpu, and individual 4kb transcripts encode for the accessory proteins Vif and Vpr. Fully spliced 1.8kb mRNAs encode the regulatory proteins Tat, Rev and the accessory protein Nef. Due to the position of the 5' splice donor all transcripts contain the highly structured 5' untranslated region (UTR). Intron-containing HIV-1 transcripts are exported from the nucleus by the regulatory protein Rev, which binds to an RNA structure in the *env* gene called the RRE (Sodroski et al., 1986; Malim et al., 1989; Malim and Cullen, 1991).

Fully spliced transcripts lack the RRE and are, therefore, exported from the nucleus by the cellular NXT/NXF1 pathway. Consequently, during early HIV-1 infection and the presence of low levels of Rev, fully spliced viral mRNA species predominate in the cytoplasm. However, later in infection when the levels of Rev are higher, both unspliced and partially spliced mRNAs are exported into the cytoplasm (Karn and Stoltzfus, 2011). Rev contains a nuclear export signal (NES) via which it mediates the export of intron-containing mRNAs by interacting with the cellular karyopherin chromosome region maintenance 1 (CRM1). CRM1 exports Rev-RRE complexes into the cytoplasm by associating with Ran GTPase, following which Rev is imported back into the nucleus (Fischer et al., 1995; Fornerod et al., 1997; Neville et al., 1997). The significance of CRM1 as an important Rev cofactor is supported by studies showing that the block to HIV-1 particle production in murine and rat cells can be rescued with human CRM1 (Swanson et al., 2004; Nagai-Fukataki et al., 2011; Sherer et al., 2011).

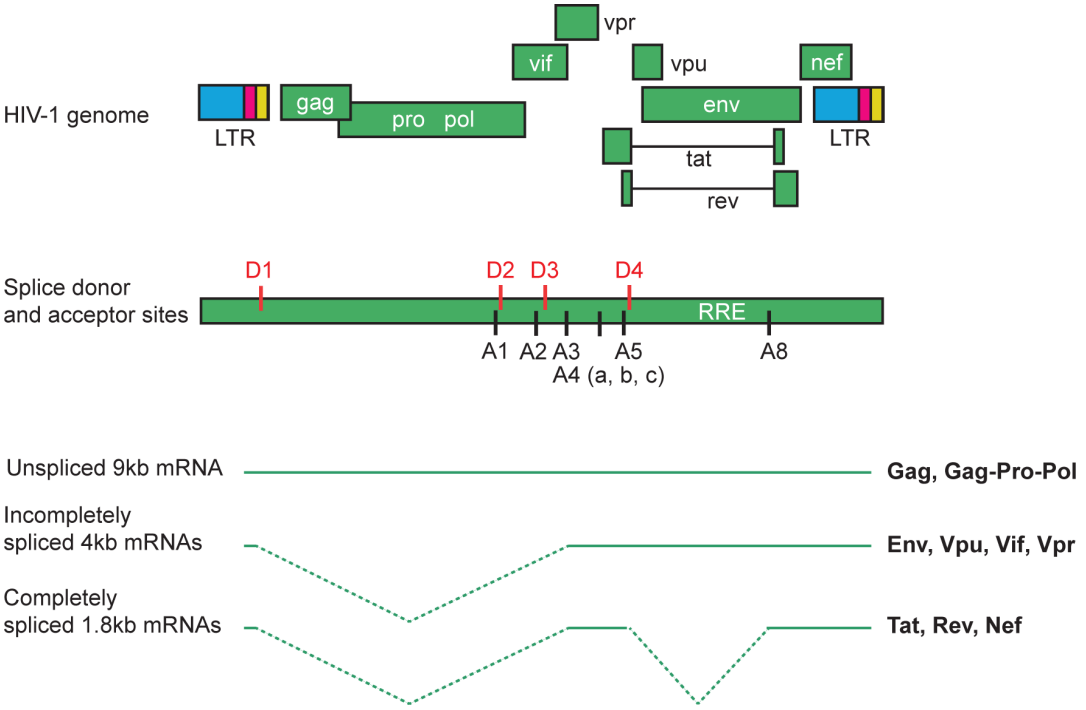


Figure 1.6. Splicing patterns of the HIV-1 genome.

The HIV-1 genome contains four splice donor (D1, D2, D3, D4; depicted in red) and eight splice acceptor sites (A1, A2, A3, A4 (a, b, c), A5, A8; depicted in black). Unspliced 9kb transcripts encode Gag and the Gag-Pro-Pol polyprotein. Incompletely spliced 4kb mRNAs encode Env, Vpu, Vif and Vpr proteins. Completely spliced 1.8kb transcripts encode the Tat, Rev and Nef proteins. Adapted from (Karn and Stoltzfus, 2011).

1.5.8 Translation and viral proteins

Retroviral transcripts contain a long and highly structured 5'UTR making them inefficient substrates for cap-dependent translation initiation (Parkin et al., 1988); nevertheless viral proteins are translated. Several cellular factors have been implicated in aiding cap-dependent translation initiation (Hartman et al., 2006; Woolaway et al., 2007; He et al., 2009; Bolinger et al., 2010; Swanson et al., 2010). Furthermore, the use of a cap-independent internal ribosome entry sequence (IRES) has also been proposed, and IRES sequences have been identified in HIV-1 although this remains controversial (Buck et al., 2001; Brasey et al., 2003).

All simple and complex retroviruses express the *gag*, *pro* and *pol* genes from unspliced mRNAs, coding for the structural and enzymatic proteins of the virus. Gag translation is initiated at an AUG codon at the 5' end of the *gag* gene till a stop codon at the 3' end of the open reading frame (ORF) that terminates translation. Some retroviruses, such as MLV, encode an additional longer Gag protein that is glycosylated, the translation of which initiates at a CUG codon upstream of the canonical AUG initiator codon. For the majority of retroviruses, a myristic acid important for membrane targeting is added to the major Gag product at the first glycine residue (Henderson et al., 1983). Gag is not only the core structural protein of virions, but is essential for packaging of the viral RNA as well as plasma membrane targeting for incorporation of the Env glycoprotein during virion budding (Goff, 2001).

The expression of *pro* and *pol* varies between viruses according to their genome organisation. In the case of gammaretroviruses, *gag*, *pro* and *pol* are in the same reading frame, however, there is a UAG stop codon between *gag* and *pro-pol* (Yoshinaka et al., 1985). Mostly, translation terminates at this stop codon, producing Gag only, however, a small percentage of the time the ribosome reads through the stop codon by inserting an amino acid such as glutamine into the chain, resulting in the production a Gag-Pro-Pol polyprotein. The presence of a downstream pseudoknot has been implicated in the mechanism of read-through (Wills et al., 1991). Lentiviruses have *gag* and *pro-pol* in separate reading frames requiring a single frameshift for translation of the Gag-Pro-Pol polyprotein. Similar to gammaretroviruses, translation results in the production of Gag predominantly, however, on occasion the ribosome slips by one nucleotide (-1) at the end of the *gag* ORF and continues translation

bypassing the stop codon in the previous reading frame (Jacks et al., 1988; Wilson et al., 1988; Parkin et al., 1992). For the betaretroviruses, *gag*, *pro* and *pol* are all in different ORFs and, therefore, two successive frameshifts are required for translation of the Gag-Pro-Pol precursor protein (Jacks et al., 1987). Pol is processed by the PR enzyme to produce the RT and IN enzymes that mediate defining steps of the retroviral life cycle (sections 1.5.3 and 1.5.5). The coordinated synthesis of Gag and Gag-Pro-Pol ensures that the correct balance of structural and enzymatic proteins is achieved in the mature virion (Jacks et al., 1988).

In the case of simple retroviruses the Env protein is translated from a fully spliced mRNA exported into the cytoplasm by the cellular NXT/NXF1 nuclear export machinery. On the other hand, HIV-1 Env is translated from a partially spliced bicistronic mRNA that also encodes Vpu by a process termed leaky scanning. The presence of a weak Kozak consensus around the *vpu* AUG initiator codon allows the ribosome to scan past it and initiate translation from the downstream *env* AUG codon (Schwartz et al., 1990; Schwartz et al., 1992).

1.5.8.1 Primate lentivirus regulatory and accessory proteins

In addition to the structural and enzymatic viral proteins, primate lentiviruses also encode regulatory proteins such as Tat and Rev, and accessory proteins such as Vif, Vpr, Vpx, Vpu and Nef. These proteins are translated from either partially spliced transcripts (Vif, Vpu, Vpr, Vpx) or fully spliced mRNAs (Tat, Rev and Nef).

The regulatory proteins Tat and Rev are dedicated to regulation of the viral life cycle, namely transcription of the viral RNA and nuclear export, respectively (sections 1.5.6 and 1.5.7). The accessory proteins have evolved to accomplish a range of functions such as evasion of the host immune response and counteraction of cellular restriction factors to enable effective replication and spread of the virus. Vif, Vpu and Vpx are prime examples of viral factors that antagonise antiviral host proteins, the functions of which will be discussed in greater detail in later sections in context with the restriction factors (section 1.8.2).

Nef is small viral accessory protein that is produced early during infection, similarly to Tat and Rev. SIVs use Nef instead of Vpu to counteract tetherin, however in addition to

this Nef is a multifunctional protein with roles in trafficking of host factors and escape from immune recognition. Nef expression downregulates CD4 from the surface of infected cells via clathrin coated pits and endosomal pathways leading to the eventual degradation of CD4 in lysosomes (Chaudhuri et al., 2007). Similarly, the HIV-1/SIVcpz Vpu accessory protein also reduces the expression of CD4 on the surface of infected cells by interacting with CD4 in the endoplasmic reticulum (ER) and triggering its proteasomal degradation (Margottin et al., 1998). Targeting of CD4 in this manner reduces the chances of superinfection, facilitates efficient virus release and in the case of Vpu-mediated degradation, prevents the formation of Env-CD4 complexes in the ER enhancing the incorporation of Env into assembling virions [reviewed in (Kirchhoff, 2010)]. Nef also reduces the surface expression of major histocompatibility complex (MHC) class I molecules on infected cells by, firstly, redirecting them from the trans-Golgi network to endosomal pathways instead of the cell surface and, secondly, downregulating MHC class I from the surface of the cell directly (Lubben et al., 2007). This serves to hamper the virus-specific CD8+ CTL response (Swigut et al., 2004). Interestingly, Nef selectively targets only human leukocyte antigen (HLA) A and B to avoid recognition and attack by natural killer (NK) cells (Cohen et al., 1999). Nef also affects antigen presentation by MHC class II (Stumptner-Cuvelette et al., 2001) and, more recently, has been reported to induce apoptosis in bystander CD4+ T cells (Lenassi et al., 2010). Furthermore, a crucial role for Nef has also emerged in the modulation of host cytoskeletal-dynamics and consequently immune activation *in vivo*. Nef partially associates with the cytoskeleton and localises to immunological synapses where it potentiates TCR signalling (Fackler et al., 1999; Fenard et al., 2005; Thoulouze et al., 2006).

Vpr is a 96 amino acid protein that is packaged into budding viral particles. Aside from the capacity of Vpr from certain SIV isolates to counteract the recently identified cellular restriction factor, sterile α motif (SAM) and HD domain containing protein (SAMHD1), Vpr expression is also able to arrest cells in the G2 phase of the cell cycle (Jowett et al., 1995; Goh et al., 1998). Reports have attributed this to the association of Vpr with the cullin4-RING ubiquitin ligase (CRL4)-DNA damage-binding protein 1 (DDB1) complex via an interaction with the DDB1-CUL4 associated factor 1 (DCAF1), which may target a cellular factor/s important for cell cycle progression to the

proteasome for degradation (Hrecka et al., 2007; Schrofelbauer et al., 2007). Vpr has also been implicated in nuclear import (section 1.5.4).

1.5.9 Assembly

Translation of the viral structural proteins initiates the assembly of progeny virions. The Gag protein consists of the N-terminal MA domain which functions in plasma membrane targeting and Env incorporation. The myristic acid, a basic patch in MA, as well as the phosphatidyl inositol (4,5) biphosphate ($PI(4,5)P_2$) lipid in the plasma membrane are required for Gag membrane targeting (Ono et al., 2004). The central CA region and NC domains mediate the protein-protein interactions for assembly of the immature virion, and NC also packages the viral genome amongst other functions. For HIV-1, the C-terminal p6 region contains the late assembly domains for viral release. HIV-1 also encodes the spacer p2 and p1 peptides that assist the conformational changes in Gag during processing and maturation (Sundquist and Krausslich, 2011). The assembly process is directed primarily by the Gag precursor and minimal regions of Gag alone can assemble immature virions (Accola et al., 2000). Experiments with HIV-1 have shown that the MA domain is dispensable for assembly provided that the myristyl switch is intact (Reil et al., 1998). Furthermore, swapping the NC domain with a leucine zipper dimerisation or tetramerisation motif allows efficient assembly, suggesting that the protein-protein interaction function of NC, but not RNA packaging, is necessary for assembly (Accola et al., 2000; Johnson et al., 2002). On the other hand, mutations in the C-terminal domain of CA and p1 are detrimental for assembly (von Schwedler et al., 2003).

In the case of gammaretroviruses and lentiviruses, the myristyl switch at the N-terminus of MA targets the Gag precursor to the inner face of the plasma membrane for assembly. In contrast, betaretroviruses such as M-PMV assemble in the cytoplasm and are subsequently transported to the plasma membrane for Env incorporation and budding. Interestingly, the substitution of a single amino acid in the MA domain of the M-PMV Gag precursor can shift the site of virion assembly to the plasma membrane (Rhee and Hunter, 1990). The presence of a dominant cytoplasmic targeting-retention signal accounts for this observation (Yasuda and Hunter, 2000; Sfakianos et al., 2003).

The Gag-Pro-Pol polyprotein, Env and, in the case of HIV-1, the accessory protein Vpr are incorporated into assembling virions by interacting with Gag. The Env precursor, gp160, is synthesised and inserted into the ER cotranslationally for glycosylation. Heavily glycosylated gp160 is assembled into trimers and transported to the Golgi where it is cleaved by the cellular protease furin into the gp120 and gp41 subunits, following which the trimers are delivered to the plasma membrane and expressed on the cell surface. Env is incorporated into virions at the site of assembly during viral budding through non-specific interactions between the cytoplasmic tail of gp41 and the MA region of Gag (Freed and Martin, 1996), which may require the cellular factor tail-interacting protein, 47 kD (Tip47) (Bauby et al., 2010). The HIV-1 accessory proteins Nef and Vif are also incorporated into assembling virions non-selectively. Cellular proteins have also been detected in HIV-1 particles, such as MHC class II molecules, antiviral APOBEC3 proteins as well as the putative RNA helicase moloney leukemia virus 10 (MOV10) (Chertova et al., 2006) (section 1.13).

1.5.9.1 RNA packaging

The dimeric unspliced viral genome is incorporated into virions through interactions between the Psi at the 5' end of the genome and the NC domain of Gag (Berkowitz et al., 1993). The Cis-His boxes and basic residues in NC are important for this interaction (Housset et al., 1993). The viral Psi is composed of a number of important stem loop structures that contribute to the overall efficiency of packaging (Fisher and Goff, 1998), and replacing one of these stem loops with a heterologous NC-binding sequence maintains efficient genome packaging demonstrating that binding to NC is the main function of these cis-acting elements (Clever et al., 2000). The current model for HIV-1 genome packaging suggests that Gag binds to the dimeric genome in the cytoplasm and subsequently travels to the plasma membrane for assembly (Jouvenet et al., 2009; Kutluay and Bieniasz, 2010). The unspliced primary transcript is also a template for the translation of Gag and Gag-Pro-Pol precursor proteins. HIV-2 was previously reported to package its genome co-translationally (Kaye and Lever, 1999), although the efficient production of HIV-2 vectors has rendered these findings somewhat controversial. On the other hand, MLV uses two separate pools for translation and assembly (Levin and Rosenak, 1976). In the case of HIV-1, studies have attempted to understand whether translation and assembly are mutually exclusive events (Butsch and Boris-Lawrie, 2000) although this is still not completely clear. Retroviral

particles also package tRNAs for the initiation of reverse transcription. The Gag-Pro-Pol precursor and genomic RNA selectively incorporate tRNAs, which are then annealed to the PBS for initiation of reverse transcription (Kleiman et al., 2010).

1.5.10 Budding

The viral lipid bilayer is derived from the cell plasma membrane during budding. Assembly is thought to occur at sites in the plasma membrane termed lipid rafts, supported by the enrichment of raft lipids in the viral membrane as well as $PI(4,5)P_2$ (Brugger et al., 2006; Chan et al., 2008). HIV-1 budding is also polarised in that virions accumulate at sites in close connection with uninfected cells for cell-to-cell transmission across a virological synapse [reviewed in (Mothes et al., 2010)].

Viral release is catalysed by the host endosomal sorting complexes required for transport (ESCRT) machinery, which bind to the 'late domain' motifs in Gag. The location and number of these motifs can vary depending on the virus. HIV-1 harbours two such motifs in the C-terminal p6 domain and the main PTAP motif recruits the tumour susceptibility gene 101 (TSG101) subunit of the ESCRT-I complex (Garrus et al., 2001; Martin-Serrano et al., 2001), whereas the second YPXL motif binds to the ESCRT-III factor ALIX (Strack et al., 2003). The p6 domain also interacts with members of the neural precursor cell expressed, developmentally down-regulated 4 (NEDD4) ubiquitin ligase family, an interaction also observed between the PPXY late domain motifs in retroviruses such as MLV (Weiss et al., 2010). Both TSG101 and ALIX interactions ultimately orchestrate the recruitment of ESCRT-III and the vacuolar protein sorting 4 (VPS4) ATPase complexes, which perform the membrane fission step [reviewed in (Weiss and Gottlinger, 2011)]. The ESCRT-III charged multivesicular body proteins (CHMPs) CHMP2 and CHMP4 are necessary for HIV-1 budding, however, CHMP1 and CHMP3 have also been reported to play a role in this process (Morita et al., 2011). CHMP4 filaments together with CHMP2 have been observed to form 'spirals' at the neck of the budding virus, which together with VPS4 mediate fission [reviewed in (Guizetti and Gerlich, 2012)]. Furthermore, the ATPase activity of VPS4 is required for disassembly and release of the ESCRT-III complex (Lata et al., 2008; Wollert et al., 2009).

1.5.11 Maturation

Immature non-infectious viral particles bud from the plasma membrane and are processed by the viral PR enzyme for the production of mature infectious virions. PR exists as a Gag-Pro-Pol fusion polyprotein in the immature particle, and is responsible for an intramolecular cleavage event to release itself from the precursor protein (Tang et al., 2008). PR then processes the Gag and Gag-Pro-Pol proteins to the smaller structural and enzymatic components MA, NC, CA, p6 in the case of HIV-1, PR, RT and IN (section 1.4). The morphology of the virion changes drastically during maturation from a spherical shape and electron-lucent centre to a more condensed shape with a characteristic core that is detached from the Env lipid bilayer (Goff, 2001). Maturation of the virus activates gp41 for fusion, assembles capsid into the characteristic core and stabilises the genomic RNA. Viruses lacking PR or the use of PR inhibitors produce progeny that are blocked at a step following entry, however, prior to reverse transcription (Kohl et al., 1988).

1.6 Endogenous retroviruses (ERVs)

Integration of the provirus into the host genome is essential for productive infection, providing a permanent template for the generation of viral transcripts. Integration into the germline permits vertical transmission of the virus, an event that has transpired on multiple occasions over millions of years in a range of species including humans, leading to the generation of endogenous retroviruses (ERVs). Approximately 8% of the human genome is derived from endogenous retroviral sequences.

The first ERVs discovered were ASLV in chickens, and MLV and MMTV in mice by a series of immunological, virological and Mendelian inheritance studies, followed closely by the discovery of the RT enzyme [reviewed in (Weiss, 2006)]. The life cycle and genetic organisation of ERVs closely resembles that of exogenous retroviruses with *gag*, *pro*, *pol* and *env* sequences flanked by LTRs; however most ERVs are defective due to the acquisition of inactivating point mutations or larger deletions during endogenisation. For this reason human ERVs (HERVs) are non-infectious, although evidence supports the possible existence of some active HERV-K elements (Turner et al., 2001). Several full-length active ERVs have, however, been identified in mice. The intracisternal A-type particle (IAP) family related to betaretroviruses are extremely active elements, present at approximately a thousand copies per cell (Kuff and Lueders,

1988) (Figure 1.7A). IAP elements have a strictly intracellular life cycle due to the absence of a complete *env* gene and the presence of a sequence in MA that targets immature particles to the ER for budding (Ribet et al., 2008). Interestingly, ERVs related to complex retroviruses, such as the lentiviruses, are rare in comparison to those derived from simple gammaretroviruses and betaretroviruses; although such endogenous sequences have recently been identified in rabbits, lemurs and sloths (Katzourakis et al., 2007; Gilbert et al., 2009; Katzourakis et al., 2009).

As expected, ERV colonisation of the host germ line is associated with a range of potential mutagenic effects. ERV insertions can affect the expression of nearby genes by modulating the transcription, splicing and stability of cellular transcripts [reviewed in (Stoye, 2012)]. However, aside from endogenous MMTV and MLV induced tumours in inbred mice, it has been difficult to correlate HERV activation as the cause or result of human diseases such as cancer, autoimmune disorders and neuropathological conditions [reviewed in (Ruprecht et al., 2008; Dreyfus, 2011; Leboyer et al., 2011)]. On the contrary, the odd beneficial outcome of ERV insertions is exemplified by the host syncytin genes that play a vital role in mammalian placenta formation, which have been derived from the capture of *env* genes from ERVs (Mi et al., 2000; Dupressoir et al., 2009). The replication of exogenous and endogenous retroelements is controlled by cellular epigenetic mechanisms and restriction factors, which will be discussed in greater detail in later sections (section 1.8).

1.7 Retrotransposons

Retrotransposons are non-LTR endogenous retroelements that make up approximately 35% of the human genome. Human retrotransposons can be divided into the long interspersed nucleotide element-1 (LINE-1) family, short interspersed nucleotide elements (SINEs) Alu and SVA, and processed pseudogenes (Table 1.3). These elements reverse transcribe via an RNA intermediate, however, the life cycle and genetic organisation of retrotransposons varies from that of retroviruses. Due to the focus of this thesis on human LINE-1 and Alu retrotransposons, these will be discussed in greater detail.

Table 1.3. Human retrotransposons.

Retrotransposon	Percentage of human genome
LINE-1	~21%
Alu	~10%
SVA	~1%
Processed pseudogene	~1%

1.7.1 LINE-1 and Alu

The LINE-1 family of retrotransposons are the only autonomously acting non-LTR elements in humans. Although the majority of LINE-1 members are inactive due to truncations and mutations, approximately 80-100 full-length retrotransposition-competent LINE-1s are active in an individual (Ostertag and Kazazian, 2001; Brouha et al., 2003; Martin et al., 2005). Members of this retrotransposon family are approximately 6kb in length with a 5' UTR and a 3' UTR flanking two ORFs separated by a small spacer (Figure 1.7B). An internal RNA pol II promoter is located in the 5' UTR and the 3' UTR contains the polyadenylation signal (Swergold, 1990). Read-through of the polyadenylation signal generates chimeric LINE-1 transcripts containing the 3' flanking host sequences (Moran et al., 1999; Goodier et al., 2000). The 5' UTR also contains an antisense promoter (ASP) that has been implicated in the post-transcriptional control of LINE-1 retrotransposition (Yang and Kazazian, 2006). ORF1 encodes a 40kda protein termed ORF1p that consists of an N-terminal coiled-coil domain required for trimerisation, a central RNA recognition motif (RRM) and a basic C-terminal domain, the latter two of which are necessary for nucleic acid binding. ORF1p also possesses nucleic acid chaperone activity important for nuclear import and subsequent reverse transcription and integration steps (Khazina and Weichenrieder, 2009; Khazina et al., 2011)[reviewed in (Martin, 2010)]. ORF2 encodes the 150kda protein called ORF2p with endonuclease (EN) and RT enzymatic activities. Reports have also shown the cysteine-rich domain at the C-terminal end to be important for retrotransposition (Mathias et al., 1991; Feng et al., 1996). ORF1p and ORF2p are translated from a bicistronic transcript, and uniquely, ORF2p is translated by an unconventional termination and reinitiation mechanism and not IRES-mediated translation (Alisch et al., 2006; Dmitriev et al., 2007). Both ORF1p and ORF2p demonstrate *cis*-preference by preferentially binding to their encoding mRNA in the

cytoplasm leading to the formation of a ribonucleoprotein (RNP) complex that serves as the equivalent of a retroviral RTC/PIC (Hohjoh and Singer, 1996; Kulpa and Moran, 2006; Doucet et al., 2010).

Alu is a human non-LTR nonautonomous retrotransposon that does not encode any proteins and is, therefore, dependent on LINE-1 ORF2p to facilitate its retrotransposition in *trans* (Dewannieux et al., 2003) (Figure 1.7B). Full-length active Alu elements are approximately 300bp in length with a polyA tail and contain two monomeric sequences derived from the 7SL RNA, which is the signal recognition particle (SRP) RNA, separated by an adenosine-rich sequence. The internal RNA pol III promoter is located in the left monomer [reviewed in (Beck et al., 2011)]. The left monomer also consists of a binding site for SRP 9/14, an interaction that is thought to associate Alu elements with ribosomes and nascent LINE-1 ORF2p.

Unlike retroviruses, retrotransposons couple reverse transcription and integration by a mechanism called target-site primed reverse transcription (TPRT) that takes place in the nucleus (Figure 1.8). As retrotransposition can occur in non-dividing cells, the RNP complex is actively imported into the nucleus (Kubo et al., 2006). Within the nucleus, ORF2p-EN nicks a single strand of the host DNA to expose a free 3' OH that serves as a primer for ORF2p-RT to initiate reverse transcription of the first cDNA strand using the mRNA as a template. Cleavage of the second host DNA strand permits generation of the second cDNA strand using the first cDNA strand as a template. The integrated LINE-1 or Alu copies are flanked by characteristic target site duplications (Cost et al., 2002; Christensen and Eickbush, 2005). Significantly, an ORF2p-EN independent mechanism of LINE-1 retrotransposition has also been proposed (Morrish et al., 2002; Morrish et al., 2007).

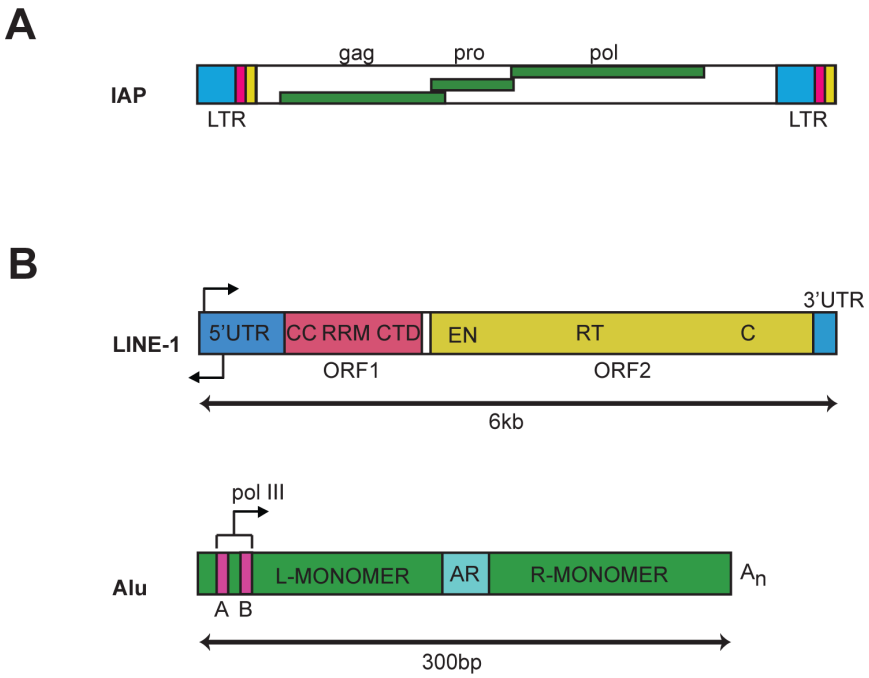


Figure 1.7. IAP, LINE-1 and Alu genome organisation.

(A) IAP genome organisation. The LTR-containing IAP endogenous retrovirus (ERV) encodes only *gag*, *pro* and *pol*. **(B) LINE-1 and Alu genome organisation.** LINE-1 is a non-LTR retrotransposon consisting of a 5' untranslated region (UTR), two open-reading frames (ORFs) and a 3' UTR. ORF1 encodes a protein with a coiled-coil domain (CC), an RNA-recognition motif (RRM) a basic carboxy-terminal domain (CTD). ORF2 encodes a protein with endonuclease (EN) and reverse transcriptase (RT) enzymatic activities, as well as a cysteine-rich carboxy-terminal region (C). The arrows in the 5' UTR depict the sense and antisense LINE-1 promoters. Alu is a non-LTR retrotransposon that is transcribed from a pol III promoter and is composed of two monomeric sequences derived from the 7SL RNA (L-MONOMER and R-MONOMER) separated by an adenosine-rich sequence (AR). Adapted from (Beck et al., 2011).

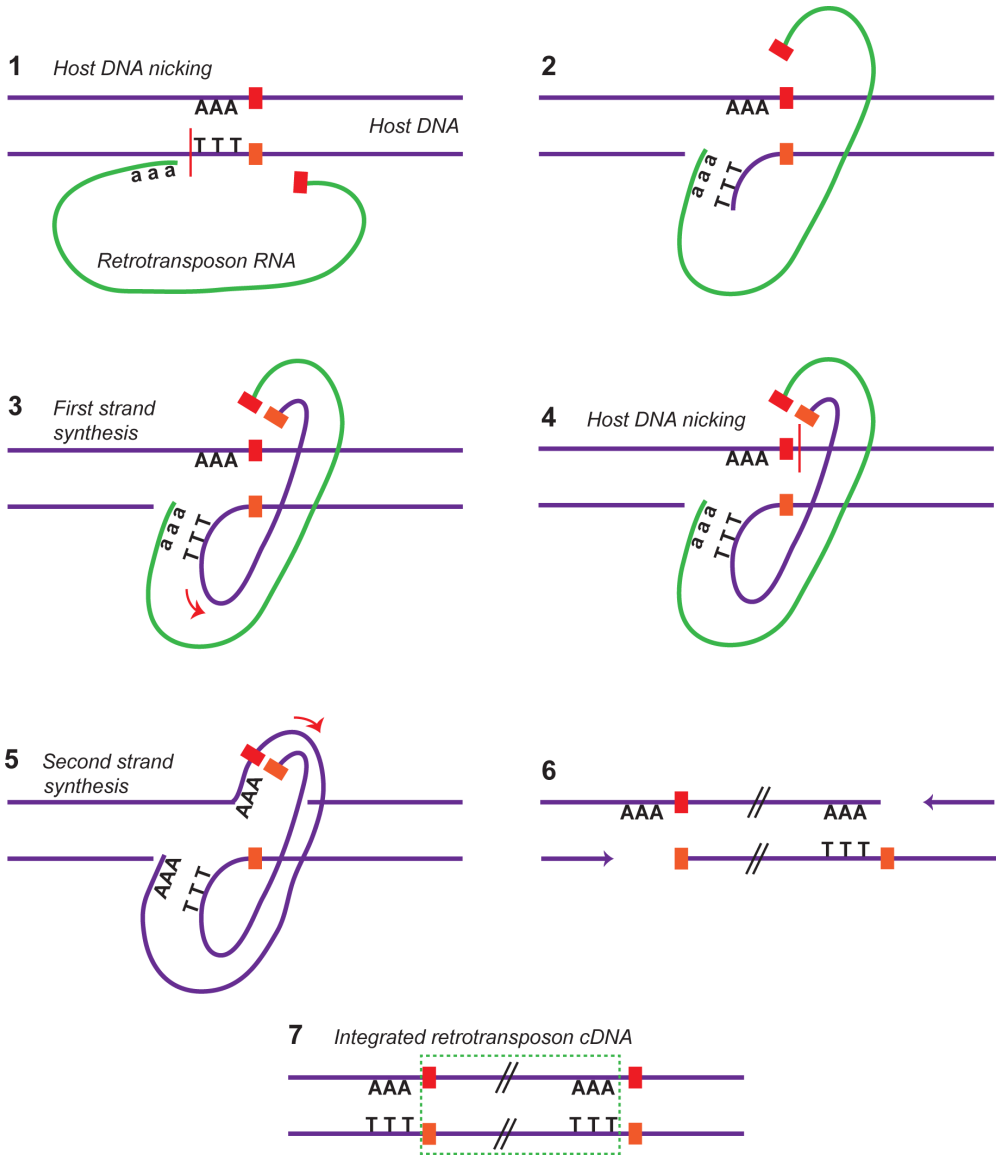


Figure 1.8. Target-primed reverse transcription.

The LINE-1 ORF2p-EN activity nicks a single strand of the host DNA (1) exposing a free 3' OH (2) that serves as a primer for initiation of reverse transcription by LINE-1 ORF2p-RT enzymatic activity and synthesis of the first cDNA strand (3). LINE-1 ORF2p-EN activity nicks the other single strand of the host DNA (4) priming synthesis of the second cDNA strand using the first cDNA strand as a template (5). Complete extension and ligation of the first and second strands (6) generates an integrated copy of the retrotransposon cDNA flanked by characteristic target-site duplications (7). DNA is illustrated in purple and RNA in green. The red and orange boxes represent sites of complementarity. Adapted from (Holmes et al., 2007).

1.7.2 Disease associations

Retrotransposition events can be mutagenic in a number of ways. Insertions can directly disrupt exons, or alternatively, integrations into introns can result in aberrant splicing of transcripts. LINE-1 retrotranspositions have also been associated with large genomic deletions and inversions [reviewed in (Kazazian and Goodier, 2002)]. Although infrequent *in vivo*, read-through of polyadenylation signals encourages the retrotransposition of cellular mRNAs, termed processed pseudogenes. Remarkably, such a retrotransposition event mediated the insertion of the CypA cDNA into the TRIM5 locus in owl monkeys, resulting in the production of a novel HIV-1 restriction factor in this species (Sayah et al., 2004). Such an event has also transpired independently in rhesus macaques, although the antiviral specificity of TRIMCyp in this species varies (Wilson et al., 2008). Furthermore, the LINE-1 ORF2p-EN activity has been proposed to cause genomic instability by mediating double-strand breaks [reviewed in (Beck et al., 2011)]. Such events can occur in both the germ line, although less likely due to strong negative selection pressures, as well as somatic cells. To date, at least 96 retrotransposition insertions have resulted in single gene diseases such as haemophilia, cystic fibrosis, breast cancer and colon cancer [reviewed in (Hancks and Kazazian, 2012)]. Somatic LINE-1 insertions have also been detected in lung, prostate and ovarian cancers (Iskow et al., 2010; Lee et al., 2012). Interestingly, LINE-1 copy numbers are elevated in the human brain in comparison with the heart and liver (Coufal et al., 2009) and, remarkably, over 20,000 novel LINE-1 and Alu insertions have been identified in the human hippocampus and caudate nucleus (Baillie et al., 2011), collectively suggesting that somatic retrotransposition events may underlie non-hereditary normal and abnormal neurobiological processes.

1.8 Regulation of exogenous and endogenous retroelements

Hosts have developed multiple transcriptional and post-transcriptional mechanisms to protect themselves from the pathogenic and mutagenic effects of exogenous and endogenous retroelements. This section will review the most prominent of these innate lines of defence.

1.8.1 Epigenetic regulation

One of the crucial ways to control the replication of retroviruses and retrotransposons is to inhibit their transcription through extensive DNA methylation at promoter sequences.

This process is orchestrated by a family of DNA methyltransferase enzymes that target a methyl group to the cytosine residue of a CpG dinucleotide. ERVs and retrotransposons in mice germ cells are targeted by DNA methylation through the nucleotide complementarity of small RNAs such as piwi-interacting RNAs (piRNAs). PIWI proteins, which are members of the Argonaute (AGO) family, drive these processes and male mice that are homozygous null for these factors display a loss of DNA methylation at retroelement promoters in germ cells and an increase in the abundance of ERV and retrotransposon transcripts (Aravin et al., 2007; Kuramochi-Miyagawa et al., 2008). Studies have also shown microRNAs (miRNAs) to control the methylation of gene promoters in mice, suggesting that other small RNA pathways may also facilitate such processes (Benetti et al., 2008; Sinkkonen et al., 2008). Alternatively, it has been known for some time that Moloney-MLV (M-MLV) replication is inhibited in embryonic carcinoma and embryonic stem cells. Recent studies have attributed this to a PBS-mediated silencing complex containing the integral transcriptional silencing factor TRIM28 (Wolf and Goff, 2007; Wolf et al., 2008; Wolf et al., 2008; Wolf and Goff, 2009). Interestingly, LINE-1 and Alu insertions have been implicated in several human cancers and their increased activity has been correlated with hypomethylation of retrotransposon promoters supporting the presence of this epigenetic regulatory pathway in humans [reviewed in (Belancio et al., 2010)]. LINE-1 expression and retrotransposition are also enhanced in the absence of the methyl-CpG-binding protein 2 (MeCP2), a factor necessary for global DNA methylation (Muotri et al., 2010). Furthermore, piRNA clusters have been identified in humans as well (Girard et al., 2006) and, more recently, a report has identified naturally occurring endogenous short-interfering RNAs (endo-siRNAs) that silence human LINE-1 expression through DNA methylation of the promoter (Chen et al., 2012).

1.8.2 Cellular restriction factors

Mammalian cells express a range of proteins that restrict the replication of exogenous and endogenous retroelements at various stages of the life cycle. Retroviruses, such as HIV-1, have also evolved a number of diverse accessory proteins to counteract these intrinsic restriction factors in favour of efficient virus replication.

1.8.2.1 Fv4

The Fv4 gene, identified in Japanese wild mice, confers resistance to infection by Friend virus (Suzuki, 1975). This gene encodes a defective provirus lacking all of *gag* and most of *pol*, although retaining a complete *env* sequence with several mutations (Ikeda et al., 1985). The mutated Env protein interacts with the E-MLV CAT-1 receptor, therefore, downregulating it from the surface and blocking infection by viruses utilising the same receptor for entry (Kai et al., 1986; Ikeda and Sugimura, 1989).

1.8.2.2 Fv1

The Fv1 locus also controls the susceptibility of certain mouse strains to Friend MLV infection (Pincus et al., 1971; Pincus et al., 1975). Two alleles of Fv1 confer resistance to particular MLVs, namely the $Fv1^b$ allele that blocks infection by N-tropic viruses and the $Fv1^n$ allele that restricts infection by B-tropic viruses; heterozygous mice can inhibit both N and B-tropic viruses. The Fv1 gene encodes a Gag-like protein related to the murine ERV-L family, and characterisation of the block revealed that restricted viruses can enter the cell and reverse transcribe, however, the PIC is trapped in the cytoplasm and does not enter the nucleus (Pryciak and Varmus, 1992). The CA protein and more specifically residue 110 determines sensitivity to Fv1, (Kozak and Chakraborti, 1996), however, the exact mechanism of action remains to be elucidated (Best et al., 1996; Benit et al., 1997).

1.8.2.3 TRIM5α and TRIMCyp

The identification of Fv1 led to the realisation that many mammalian cell lines, including human, could also restrict N-tropic MLVs. Similar to Fv1, susceptibility of the virus to restriction was dependent on amino acid 110 of the CA protein, although the block to infection occurred prior to reverse transcription (Towers et al., 2000). Furthermore, nonhuman primate cell lines restricted HIV and SIV with similar characteristics (Cowan et al., 2002; Munk et al., 2002; Hatziioannou et al., 2003). A screen for HIV-1 restriction factors by introducing a cDNA expression library from non-permissive rhesus macaque cells into permissive human cells resulted in the identification of a novel restriction factor TRIM5α (Hatziioannou et al., 2004; Keckesova et al., 2004; Stremlau et al., 2004).

TRIM5α is a member of the 'tripartite motif'-containing family of proteins, which consist of an N-terminal RING and B-box type 2 domain, a central coiled-coil domain for dimerisation, and in the case of TRIM5α a C-terminal domain called the B30.2 or PRYSPRY domain (Malim and Bieniasz, 2012). Evolutionary and mutagenesis studies have deduced that the SPRY domain is responsible for the recognition of specific retroviral CA proteins (Sawyer et al., 2005; Stremlau et al., 2005; Yap et al., 2005). The restrictive activity of TRIM5α is fairly broad and dependent on the species of origin, for example, human TRIM5α can restrict N-MLV and equine infectious anaemia virus (EIAV), but not HIV-1, however, TRIM5α from Old World monkeys can restrict HIV-1 (Hatziioannou et al., 2004; Keckesova et al., 2004; Stremlau et al., 2004). This capacity of TRIM5α proteins to 'tolerate' viruses that naturally infect the host in comparison to the restriction of viruses from other species is a potential mechanism for the prevention of cross-species transmission events.

Lentiviral CA proteins also bind to the host protein CypA, and this interaction has been shown to increase the sensitivity of HIV-1 to TRIM5α-mediated restriction in Old World monkeys, but not in human cells (Berthoux et al., 2005; Keckesova et al., 2006). Alternatively, CypA has a stimulatory effect on HIV-1 infectivity in human cells possibly by acting as a cofactor, or alternatively, counteracting an unidentified antiviral factor (Sokolskaja et al., 2006). Remarkably, in owl monkeys and rhesus macaques, retrotransposition events have transduced the CypA cDNA into the TRIM5α locus generating a chimeric gene encoding the TRIMCypA fusion protein in which CypA has replaced the PRYSPRY domain for capsid binding (Sayah et al., 2004). Although the mechanism by which TRIM5α and TRIMCypA restrict retroviruses is not completely understood, studies suggest that accelerated disruption of the viral core may be an important factor (Stremlau et al., 2006; Wilson et al., 2008).

1.8.2.4 ZAP

The zinc-finger antiviral protein (ZAP) was identified in a rat cDNA library screen as an MLV restriction factor able to block the accumulation of viral RNAs in the cytoplasm, although transcribed viral transcripts could be readily detected in the nucleus (Gao et al., 2002). Transfer of a small sequence from the 3' end of the viral RNA to heterologous cellular mRNAs confers susceptibility to ZAP-mediated downregulation.

Studies have shown that ZAP binds to viral RNAs via its zinc-finger clusters targeting them for degradation via the RNA exosome (Guo et al., 2004; Guo et al., 2007).

1.8.2.5 Tetherin

A common experimental approach for the identification of cellular restriction factors is the fusion of cells permissive and non-permissive to viral infection or steps in the viral life cycle, to form heterokaryons that express factors from both cell types. A dominant restrictive phenotype suggests the presence of an intrinsic restriction factor in the non-permissive cell type and, alternatively, a susceptible phenotype supports the presence of a cofactor in permissive cells that is absent in non-permissive cells. The HIV-1 accessory protein Vpu was shown to be required for HIV-1 infection in heterokaryon experiments that revealed a dominant block to HIV-1 release in a type I interferon α (IFNα)-sensitive manner (Varthakavi et al., 2003; Neil et al., 2007). HIV-1 particles remained trapped or tethered to the surface of infected cells in the absence of Vpu, following which they were internalised into endosomes (Neil et al., 2006). Subsequently, the IFNα-inducible restriction factor tetherin was identified by microarray studies (Neil et al., 2008; Van Damme et al., 2008).

Tetherin is a type II transmembrane protein that functions as a dimer. It contains an N-terminal cytoplasmic and transmembrane domain, an extracellular coiled-coil domain necessary for dimerisation, and a C-terminal glycophosphatidylinositol (GPI) lipid anchor that is inserted back into the plasma membrane. The configuration of tetherin rather than its primary sequence is important for antiviral activity, which also explains its capacity to restrict a diverse range of enveloped viruses (Perez-Caballero et al., 2009). However, the N-terminal transmembrane domain and GPI anchor are essential for its tethering ability, one pair of which inserts itself into the viral lipid envelope and the other pair remains attached to the plasma membrane (Malim and Bieniasz, 2012).

Considering the broad antiviral activity of tetherin, HIVs and SIVs have evolved independent mechanisms to antagonise it. HIV-1 Vpu is a small 14kda protein with a transmembrane domain and short cytoplasmic tail. Vpu counteracts tetherin by binding to it and inducing its cell surface downregulation and ultimate degradation (Van Damme et al., 2008; Mitchell et al., 2009; Dube et al., 2010). The Vpu transmembrane

domain and cytoplasmic tail determinants necessary for the antagonism of tetherin have been mapped (Vigan and Neil, 2010; Kueck and Neil, 2012). The majority of SIVs do not encode Vpu and have instead resorted to using Nef for the counteraction of tetherin, which is thought to occur via the AP-2 clathrin adaptor complex (Zhang et al., 2009; Zhang et al., 2011). Additionally, primate lentiviruses such as HIV-2 utilise the Env protein for tetherin antagonism (Le Tortorec and Neil, 2009). Interestingly, human tetherin lacks a five-residue Nef-sensitivity motif in its cytoplasmic domain likely resulting in the exchange of tetherin counteraction activity to Vpu and Env, respectively, following the zoonotic transfer of SIVcpz and SIVsmm into humans (Sauter et al., 2009; Lim et al., 2010). The inability of HIV-1 groups O and P to adapt to human tetherin like group M viruses may account for their limited contribution to the global HIV-1 pandemic (Sauter et al., 2011; Yang et al., 2011).

1.8.2.6 SAMHD1

HIV-1, however, not HIV-2/SIVsmm infection is inherently blocked in DCs and is inefficient in macrophages, an effect that was shown to be bypassed by providing the HIV-2/SIVsmm specific accessory protein Vpx in trans (Goujon et al., 2008; Sharova et al., 2008). Recently, biochemical screens identified the 626 amino acid IFN-inducible protein SAMHD1 as the myeloid cell restriction factor counteracted by Vpx (Hrecka et al., 2011; Laguette et al., 2011). Vpx binds and targets SAMHD1 for proteasomal degradation via the CRL4-DCAF1 E3 ubiquitin ligase complex (Hrecka et al., 2011; Ahn et al., 2012). Vpx arose as a duplication of the Vpr gene, and is found in only two of eight major primate lentivirus lineages; however, Vpr is present in all lineages. Interestingly, some Vpr proteins can also counteract SAMHD1, and evolutionary studies provide evidence for the neofunctionalisation of Vpr to degrade SAMHD1 prior to the existence of Vpx (Lim et al., 2012).

Aicardi-Goutières syndrome (AGS) is an autoimmune disease that affects individuals harbouring mutations in SAMHD1, characterised by the mimicking of congenital infections, an increase in IFNα production and symptoms reminiscent of systemic lupus erythematosus (SLE). This supports a role for SAMHD1 in the negative regulation of IFNα production and immune modulation (Rice et al., 2009). Mutations in the 3' exonuclease three prime repair exonuclease 1 (TREX1) and the RNase H2 endonuclease

complex also contribute to AGS pathogenesis (Crow et al., 2006; Crow et al., 2006). Considering the functions of these factors in nucleic acid metabolism, it is not unlikely that SAMHD1 may also operate in a similar pathway. Consistently, studies have shown SAMHD1 to be a dGTP-regulated dNTP triphosphohydrolase, implying that SAMHD1 may restrict viruses by depleting the intracellular pool of nucleotides, therefore, impeding viral cDNA synthesis (Goldstone et al., 2011; Powell et al., 2011). Interestingly, the fact that HIV-1 has not acquired a factor to counteract SAMHD1, similar to HIV-2/SIVsmm, suggests that the counteraction of SAMHD1 is dispensable for HIV-1 replication. Furthermore, recognising the role of SAMHD1 in immune regulation, not counteracting SAMHD1 may even be beneficial for the virus.

1.8.2.7 TREX1

Sensing of foreign nucleic acids by toll-like receptors (TLRs) and cytosolic sensors elicits a potent type I IFN response, a principal that underlies antiviral immune responses [reviewed in (Stetson and Medzhitov, 2006)], but can also result in autoimmune diseases such as AGS (Crow et al., 2006). As discussed, mutations in TREX1 are associated with AGS and, furthermore, studies in TREX1-deficient mice exhibit a potent decrease in survival due to inflammatory myocarditis (Morita et al., 2004). TREX1 has been reported to metabolise reverse transcribed DNA derived from multiple endogenous retroelements and inhibit the retrotransposition of LINE-1 and IAP when overexpressed (Stetson et al., 2008). More recently, a human endogenous retrovirus has been suggested to trigger Chilblain lupus, an autoimmune disease linked with TREX1 deficiency (Perl et al., 2010). Furthermore, treating TREX1 knockout myocarditis mice with RT inhibitors resolves disease (Beck-Engeser et al., 2011). These studies strongly implicate TREX1 in the control of endogenous retroelement replication and their potential contribution to autoimmunity (Stetson et al., 2008). Highlighting the complexity of host-pathogen relationships, the nucleic acid metabolising function of TREX1 is necessary for the efficient spread and replication of HIV-1 since TREX1 digests excess reverse-transcribed HIV-1 DNA preventing the induction of a type I IFN response (Yan et al., 2010). Furthermore, the SET complex, which includes TREX1, supports HIV-1 infection by preventing autointegration and, therefore, facilitating integration into the host chromosomal DNA (Yan et al., 2009).

1.8.2.8 APOBEC3 proteins and HIV-1

Vif is a 23kda accessory protein encoded by all primate lentiviruses. Vif was shown to be required for HIV-1 replication in primary cells and also some immortalised cell lines such as HUT78 and CEM, yet was dispensable for others such as SupT1 and CEM-SS (Gabuzda et al., 1992; von Schwedler et al., 1993). Heterokaryon experiments revealed a dominant restrictive phenotype that could be counteracted by Vif (Simon et al., 1998), following which cDNA subtraction experiments searching for factors expressed in non-permissive cells but not permissive cells identified APOBEC3G (A3G), a member of the apolipoprotein B mRNA editing enzyme polypeptide like 3 (APOBEC3) family of proteins, as the restriction factor able to inhibit *vif*-deficient virus (Sheehy et al., 2002).

The APOBEC family is composed of eleven members in humans each of which contain one or two copies of a cytidine deaminase (CDA) domain that edits cytidine residues to uridine residues in an RNA or DNA substrate. A3G consists of two CDA domains, the C-terminal deaminase domain of which mediates deamination. The N-terminal deaminase domain is not catalytically active although it is necessary for A3G packaging into nascent virions and Vif recognition (Navarro et al., 2005; Newman et al., 2005; Huthoff and Malim, 2007). In the absence of Vif, A3G is incorporated into virions through interactions between the N-terminal CDA domain and the NC region of Gag bridged by interactions between A3G and RNA (Luo et al., 2004; Wang et al., 2007; Bogerd and Cullen, 2008). Oligomerisation of A3G has been reported to be important for packaging and antiviral function as well (Huthoff et al., 2009). Following infection of target cells, A3G associates with the RTC and preferentially deaminates the third cytosine residue in 5'-CC<u>C</u>A sequences that register as guanosine-to-adenosine (G-to-A) hypermutations in the plus strand DNA, leaving the genome compromised and the virus non-infectious (Harris et al., 2003; Mangeat et al., 2003; Bishop et al., 2004).

Studies have also revealed an editing-independent mechanism of A3G antiviral activity, whereby deaminase-deficient mutant A3G proteins can still inhibit HIV-1 infection (Newman et al., 2005). Wild-type A3G reduces the accumulation of viral cDNA, originally believed to be a consequence of degradation of uridine-containing DNA by host DNA repair enzymes, although this theory has been disproved (Langlois and Neuberger, 2008). Instead A3G may physically block RT translocation along the viral RNA template (Iwatani et al., 2007; Bishop et al., 2008).

Vif binds to A3G and targets it to the cellular cullin5-elonginBC-core-binding factor β (CBFβ) ubiquitin ligase complex for polyubiquitination and proteasomal degradation, preventing its packaging into assembling virions (Sheehy et al., 2003; Yu et al., 2003; Jager et al., 2011; Zhang et al., 2011). Recognition of A3G by the N-terminal region of Vif determines the species-specific regulation of A3G by HIV and SIV Vif, a process with implications in the zoonotic transfer of SIVs into humans (Gaddis et al., 2004; Schrofelbauer et al., 2006; Russell and Pathak, 2007).

APOBEC3F (A3F) is also a double CDA domain containing APOBEC3 protein that inhibits HIV-1 infection and is antagonised by Vif similar to A3G, although the antiviral activity of A3F is less potent than that observed for A3G (Bishop et al., 2004; Liddament et al., 2004; Wiegand et al., 2004; Holmes et al., 2007). The preferred target site for A3F deamination is a 5'-T<u>C</u> instead of a 5'-C<u>C</u>. The antiviral activity of remaining APOBEC3 proteins has also been investigated although the relevant contribution of these factors *in vivo* is questionable. APOBEC3A (A3A) consists of a single CDA domain and has been implicated as the factor partially responsible for the resistance of immature monocytes to HIV-1 infection (Peng et al., 2007). A3A also deaminates incoming foreign double-stranded DNA in producer cells resulting in its degradation (Stenglein et al., 2010). Overexpression of APOBEC3B (A3B), a double CDA domain containing protein, and APOBEC3D/E (A3D/E), a single CDA domain containing protein, in single-cycle infectivity assays inhibits HIV-1 infection; however, this effect is only modest for A3D/E, and A3B is expressed at extremely low levels in CD4+ T cells probing its impact in natural infection (Bishop et al., 2004; Doehle et al., 2005; Dang et al., 2006; Bogerd et al., 2007). Alternatively, the single CDA domain containing APOBEC3C (A3C) protein is expressed in CD4+ T cells and has been reported to introduce low-level G-to-A mutations in some HIV-1 strains (Bourara et al., 2007). APOBEC3H (A3H) consists of a single CDA domain and up until recently only A3H haplotype II was shown to suppress HIV-1 infection as only this allele encoded a stable protein (OhAinle et al., 2008; Harari et al., 2009); however recently, additional A3H haplotypes were identified that also express stable proteins and inhibit HIV-1 infection (Wang et al., 2011). Studies suggest that similar to A3G and A3F, A3D/E is also sensitive to degradation by Vif, whereas A3H is only partially sensitive and A3B is resistant to Vif activity. Taking into consideration sensitivity to Vif, relative expression

levels required for restriction and side-by-side comparisons of all APOBEC3 proteins, evidence suggests that in addition to A3G and A3F only A3H and A3D/E significantly inhibit HIV-1 replication (Hultquist et al., 2011). G-to-A hypermutations are readily detected in HIV-1 infected patients suggesting that the counteraction of APOBEC3 proteins by Vif *in vivo* may be variable and incomplete. Current studies are focusing on the importance of this balance in determining the course of HIV-1 infection and progression to AIDS [reviewed in (Malim, 2009)].

1.8.2.9 APOBEC3 proteins and other retroviruses

The antiviral activity of APOBEC3 proteins extends beyond primate lentiviruses. MLV is restricted by human A3G and A3B, but not by the only murine APOBEC3 (mA3) protein. This effect that may be attributed to inefficient packaging of mA3 into MLV particles, cleavage of mA3 by the viral protease and/or resistance of MLV to mA3 deaminase activity by an unknown mechanism (Bishop et al., 2004; Doehle et al., 2005; Abudu et al., 2006; Rulli et al., 2008). Similarly, M-PMV can be inhibited by mA3, which is incorporated efficiently into nascent virions, however, is resistant to the host rhesus macaque A3G (rA3G) by selectively excluding it from particles (Doehle et al., 2006). The exact mechanism/s by which Vif-lacking simple retroviruses overcome restriction by APOBEC3 proteins requires further investigation. Interestingly, the complex retrovirus HTLV-1 is relatively resistant to A3G by preventing its incorporation into virions through the activity of an acidic patch at the C-terminus of the NC domain (Derse et al., 2007).

ERVs are active and mobile in the mouse genome, and have also been identified as targets for APOBEC3 deaminase-dependent activity; although A3A has been shown to restrict IAP replication by a packaging and editing-independent mechanism (Esnault et al., 2005; Bogerd et al., 2006; Esnault et al., 2006; Esnault et al., 2008). Interestingly, genetic studies have also discovered evidence for human A3G activity against HERVs (Armitage et al., 2008; Lee et al., 2008).

1.8.2.10 APOBEC3 proteins and retrotransposons

APOBEC3 proteins play a significant role in the innate restriction of retrotransposons as well. All human APOBEC3 proteins have been reported to variably inhibit LINE-1 retrotransposition, with A3A and A3B exhibiting the most potent activity (Bogerd et al.,

2006; Muckenfuss et al., 2006; Kinomoto et al., 2007; Niewiadomska et al., 2007). Furthermore, silencing of endogenous A3B in cell lines and human embryonic stem cells enhances LINE-1 retrotransposition (Wissing et al., 2011). A3A, A3B, A3C and A3G also restrict Alu retrotransposition in an ORF1p-independent manner (Bogerd et al., 2006; Hulme et al., 2007). Reports have demonstrated that APOBEC3 proteins suppress LINE-1 and Alu retrotransposition by an editing-independent mechanism, and also observed a decrease in the accumulation of LINE-1 cDNA (Stenglein and Harris, 2006; Hulme et al., 2007).

1.9 APOBEC3 proteins and ribonucleoprotein (RNP) complexes

Targeting and editing of viral DNA is an established activity of APOBEC3 proteins. Furthermore, a cellular function of APOBEC3 proteins is likely the regulation of endogenous retroelements. However, whether these proteins can target cellular sequences and, if so, the mechanism by which host chromosomal DNA is protected from deleterious mutations is still unclear. A3G associates with high-molecular-mass (HMM) RNP complexes in activated CD4+ T cells where its enzymatic activity is inhibited and, alternatively, is found within low-molecular-mass (LMM) RNP complexes in resting CD4+ T cells where it is enzymatically active (Chiu et al., 2005). Therefore, localisation of APOBEC3 proteins in the cytoplasm and sequestration into RNP complexes may be one such means for regulation of these proteins. Mass spectrometry approaches have identified a panel of A3G and A3F-interacting RNA-binding proteins (RBPs), which also includes MOV10 (Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). These associate with A3G in, predominantly, an RNA-dependent manner, although their interaction with A3F is less sensitive to RNase digestion (Kozak et al., 2006; Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). Several of these RBPs are mRNA processing body (P body) and stress granule (SG) factors, which are nonmembraned cytoplasmic RNP microdomains involved in the storage or degradation of translationally silenced mRNAs (section 1.10 and 1.11). Furthermore, A3G and A3F co-localise with these factors in P bodies and SGs (Wichroski et al., 2006; Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008) and A3G-RNA complexes have also been reported to shuttle between polysomes and SGs (Kozak et al., 2006). Localisation of APOBEC3 proteins to P bodies correlates with their incorporation into virions and antiviral activity, which may implicate these cytoplasmic foci in the regulation of APOBEC3 function; however, depletion of P

bodies through knockdown of P body factors DEAD (Asp-Glu-Ala-Asp) box helicase 6 (DDX6) and Lsm1 was shown to have no effect on the packaging of APOBEC3 proteins into virions or their ability to restrict HIV-1 infection (Phalora et al., 2012). Therefore, the functional relevance of the localisation of A3F and A3G to P bodies and SGs, if any, remains to be characterised and may be relevant for an alternative cellular function. Interestingly, A3G sequesters Alu RNA in HMM A3G RNP complexes preventing its association with the L1-ORF2p machinery, which may be a potential mechanism for A3G-mediated restriction of Alu retrotransposition (Chiu et al., 2006).

1.10 Cytoplasmic mRNA processing bodies (P bodies)

Gene expression is regulated by a balance between mRNA translation and decay. Degradation of the mRNA is mediated by deadenylation and removal of the 5' cap by the decapping enzymes DCP1a/DCP2, following which exoribonuclease 1 (XRN1) digests the mRNA in a 5' to 3' direction. For this to occur the nontranslating mRNA containing RNP complex (mRNP) must interact with the deadenylation/decapping machinery to form an mRNP/deadenylation/decapping complex that localises to cytoplasmic foci called P bodies [reviewed in (Parker and Sheth, 2007). Alternatively, translating mRNPs associated with polysomes are cytoplasmically diffuse. Therefore, P bodies are composed of aggregates of translationally repressed mRNPs in conjunction with the decay machinery, including the decapping enzymes DCP1a/DCP2, activator of decapping DDX6, the Lsm1-7 complex, the XRN1 exonuclease and the CCR4/POP2/NOT deadenylase complex (Ingelfinger et al., 2002; van Dijk et al., 2002; Cougot et al., 2004). In addition, factors associated with small RNA-mediated post-transcriptional RNA silencing pathways, such as the AGO proteins, GW182, MOV10, as well as the nonsense-mediated decay (NMD) factor Upf1 and antiviral proteins A3F and A3G also localise to P bodies (Eystathioy et al.; Lykke-Andersen, 2002; Liu et al., 2005; Meister et al., 2005; Wichroski et al., 2006)

P bodies are disrupted with RNase treatment signifying the important role of mRNAs in P body assembly (Teixeira et al., 2005). Blocking mRNA turnover by inhibiting translation initiation increases the size and number of P bodies and, in contrast, inhibiting translation elongation whereby nontranslating mRNAs are trapped with ribosomes decreases both the size and number of P bodies (Teixeira et al., 2005). Protein components likely contribute to P body assembly as well. In mammalian cells

knockdown of GW182, DDX6, Lsm1, Lsm4 and Ge1 drastically depletes P bodies (Yu et al., 2005). Some of these factors consist of a glutamine/asparagine (Q/N) rich prion-like domain allowing self-aggregation of proteins implicating these domains in P body formation (Reijns et al., 2008). Alternatively, individual P body components that function in translation repression pathways such as the AGO proteins, GW182, Upf1 and DDX6 (Pillai et al., 2004; Coller and Parker, 2005; Isken et al., 2008; Zipprich et al., 2009) may contribute to P body assembly indirectly by increasing the pool of translationally repressed mRNPs for aggregation.

The fate of nontranslating mRNAs in P bodies is thought to be dual, whereby they may be degraded or stored and returned to translation. Besides the localisation of mRNA decay machinery to P bodies, inhibiting mRNA turnover by blocking deadenylation dramatically decreases the size of P bodies, however, they increase in size if mRNA turnover is blocked at the stage of decapping (Sheth and Parker, 2003). Yeast studies have identified a reciprocal shuttling of mRNAs between P bodies and polysomes dependent on the translation state, (Brengues et al., 2005; Bhattacharyya et al., 2006), however, this has been proposed to be a selective process targeting only a limited population of mRNAs, and not a widespread phenomenon (Arribere et al., 2011).

1.10.1 P bodies and retroelements

The association of P bodies with retroelement life cycles is not surprising given the role of these cytoplasmic foci in RNA metabolism. The yeast retrotransposons Ty1 and Ty3 are members of the copia-like and gypsy-like family of endogenous retroelements. Genetic screens identified the Lsm1-7 complex and Dhh1 (yeast ortholog of DDX6) as being required for efficient Ty1 and Ty3 retrotransposition. Ty3 transcripts, proteins and virus-like particles (VLPs) have been shown to localise to P bodies, and the disruption of essential P body components was reported to alter the subcellular distribution of these components (Beliakova-Bethell et al., 2006). Mutations in the Ty3 Gag NC domain resulted in the mislocalisation of Gag and defective VLP assembly suggesting that P bodies may function in Ty3 assembly and/or maturation (Larsen et al., 2008).

Studies also propose a link between P bodies and retroviral replication although these are more controversial, and conclude both positive and negative regulatory roles for P

bodies. The highly conserved RNA helicase DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked (DDX3), the yeast ortholog of which accumulates in P-bodies, has been reported to be required for the nuclear export of unspliced HIV-1 transcripts via the CRM1/Rev/RRE nuclear export pathway (Yedavalli et al., 2004). Knockdown of DDX3 completely abrogated the export of intron-containing HIV-1 mRNA and effectively inhibited HIV-1 replication without affecting cell viability (Yedavalli et al., 2004; Ishaq et al., 2008). This has subsequently led to the discovery of small molecule inhibitors of DDX3 as a potential novel anti-HIV therapy (Garbelli et al., 2011; Radi et al., 2012). However, human DDX3 cannot be detected in P bodies suggesting that the stimulatory role of this protein in the HIV-1 life cycle is independent of P bodies. Reed et al recently proposed a function for DDX6 in HIV-1 Gag assembly independent of viral RNA packaging (Reed et al., 2012) and, similarly, DDX6 has been reported to be necessary for efficient packaging of the foamy virus genome (Yu et al., 2011). In contrast, the intrinsic antiviral factors A3F and A3G localise to P-bodies (Wichroski et al., 2006; Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008), although Phalora et al verified no functional relevance for this localisation in terms of APOBEC3 protein-mediated restriction of HIV-1 (Phalora et al., 2012). A study has also reported silencing of core P-body components DDX6, GW182, Lsm1 and XRN1 to enhance HIV-1 infection and, furthermore, knockdown of DDX6 in peripheral blood mononuclear cells (PBMCs) derived from HIV-1 infected patients on HAART to reactivate the virus (Chable-Bessia et al., 2009). However, Phalora et al recently confirmed no effect of P body depletion through knockdown of DDX6 and Lsm1 on HIV-1 replication (Phalora et al., 2012). Interestingly, Lu et al observed an increase in IAP expression, retrotransposition, Gag protein abundance and accumulation of reverse transcript products following depletion of P bodies by knockdown of DDX6 or the eukaryotic initiation factor E transporter (eIF4E-T)(Lu et al., 2011).

1.10.2 P bodies and other RNA viruses

A role for P bodies in the replication of other positive-strand RNA viruses has also been reported. The brome mosaic virus (BMV) depends on DDX6, the Lsm1-7 complex and DDX3 for translation of viral transcripts and also for genome replication, which likely occurs in P bodies (Noueiry et al., 2003; Mas et al., 2006). Several reports have also highlighted a positive association between hepatitis C virus (HCV) and P bodies. The HCV core protein interacts with DDX3 and silencing of DDX3 decreases HCV

replication (Ariumi et al., 2007). Again, as human DDX3 does not localise to P bodies the role of this protein in HCV infection is likely independent of P bodies. Nevertheless, HCV infection also hijacks the P body components DDX6, Lsm1, XRN1 and AGO2, but not DCP2, and redistributes these to viral production factories around lipid droplets for HCV replication (Ariumi et al., 2011). Interestingly, although P body factors are essential for HCV replication, disruption of P bodies through the knockdown of a non-essential P body factor has no effect on replication; this suggests that individual P body components and not the microdomains themselves are required for HCV infection (Perez-Vilaro et al., 2012). On the contrary, poliovirus infection induces the rapid degradation of P body components XRN1, DCP1a and the deadenylase subunit Pan3, ultimately resulting in the disruption of P bodies (Dougherty et al., 2011).

1.11 Stress granules (SGs)

SGs are cytoplasmic aggregates of stalled 48S translation initiation complexes that store translationally repressed mRNAs and form under conditions of cellular stress. SGs are defined by their inclusion of 40S ribosomal subunits and eukaryotic translation initiation factor (eIF) 2, eIF3, eIF4A, eIF4B, eIF4E, eIF4G and eIF5 (Kedersha et al., 2002), as well as RBPs such as the T-cell restricted intracellular antigen 1 (TIA-1), TIA-1-related protein (TIAR), RasGAP SH3-domain binding protein 1 (G3BP1), histone deacetylase (HDAC) and Staufen1 [reviewed in (White and Lloyd, 2012)]. P body components such as the AGO proteins, MOV10, A3F and A3G can also redistribute to SGs during conditions of cellular stress, such as heat shock. SGs are thought to represent an intermediate phase between actively translating polysomes and mRNA degrading P bodies, whereby mRNAs stored in SGs can be released for translation or alternatively targeted to P bodies for decay (Kedersha et al., 2000; Kedersha et al., 2005), although the role of SGs and their association to polysomes and P bodies is still controversial (Mollet et al., 2008). Furthermore, SGs and P bodies have also been observed to physically associate, which may facilitate potential exchanging of mRNAs (Kedersha et al., 2005).

SGs can assemble in a number of ways, although a key mechanism involves phosphorylation of eIF2α by the eIF2 kinases protein kinase R (PKR), protein kinase RNA-like endoplasmic reticulum kinase (PERK), general control nonrepressed 2

(GCN2) or heme-regulated inhibitor kinase (HRI). These are activated by various forms of cellular stress such as viral infection, oxidative stress and nutrient starvation. Phosphorylation of eIF2α reduces formation of the eIF2-GTP-$tRNA^{Met}$ ternary complex that is essential for the loading of $tRNA^{Met}$ onto the 40S ribosomal subunit for translation initiation. Instead, TIA-1 and TIAR are assembled forming noncanonical pre-initiation complexes (Kedersha et al., 1999). Subsequently, SGs can form through self-aggregation and post-translational modification of SG components. TIA-1 and TIAR consist of three RRMs followed by a glutamine rich prion-like domain at the C-terminal end. Self-oligomerisation of TIA-1 or TIAR may play a significant role in early SG assembly (Gilks et al., 2004). Furthermore, G3BP1 can also self-aggregate in a phosphorylation dependent manner (Tourriere et al., 2003). Post-translational modification of proteins, such as O-linked-N-acetylglucosamine (O-Glc-Nac) modification of ribosomal proteins also contributes to SG formation (Ohn et al., 2008).

1.11.1 SGs and retroelements

Akin to P bodies, SGs have been proposed to play both inhibitory and stimulatory roles in the replication of several RNA viruses and retrotransposons. As stated previously, the antiviral proteins A3F and A3G are relocalised from P bodies to SGs during conditions of cellular stress (Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). The nuclear RBP src-associated in mitosis, 68 kDa (Sam68) is recruited to SGs through interactions with TIA-1 (Henao-Mejia and He, 2009). The induction of SGs through Sam68 cytoplasmic mutants suppresses the expression of HIV-1 Nef, a phenomenon that correlates with the enrichment of Nef transcripts in SGs and a reduction in the downregulation of cell surface MHC class I and CD4 molecules (Henao-Mejia et al., 2009). Similarly, the HTLV-1 regulatory protein Tax has been reported to interact with the essential SG component HDAC6 to inhibit the formation of SGs (Kwon et al., 2007; Legros et al., 2011). In contrast, the SG component Staufen1 preferentially forms novel RNP granules instead of SGs during HIV-1 infection to which HIV-1 Gag and viral RNA localise, and depletion of Staufen1 results in defective assembly of viral particles and genome packaging (Abrahamyan et al., 2010). Likewise, SGs have also been implicated in the replication of retrotransposons. LINE-1 ORF1p has been observed to form SGs and interact as well as co-localise with SG components such as the Y-box-binding protein 1 (YB-1), heterogeneous nuclear ribonucleoprotein

A1 (hnRNPA1) and AGO2 (Goodier et al., 2007). Moreover, the formation of LINE-1 RNP complexes consisting of the LINE-1 RNA, ORF1p and ORF2p is essential for retrotransposition and, interestingly, these have been observed to localise to cytoplasmic microdomains associated with SGs (Doucet et al., 2010).

1.11.2 SGs and other RNA viruses

Poliovirus induces the formation of SGs during early infection in favour of translation of viral proteins, however, these are dispersed in later stages of infection through the cleavage of G3BP1 by the viral 3C proteinase (White et al., 2007). It was initially thought that TIA-1 SGs were resistant to disassembly by poliovirus infection, however, later studies revealed these to be devoid of essential SG-defining components, such as translation initiation factors (White and Lloyd, 2011). Similarly, HCV infection also induces SGs, but inhibits their formation as infection proceeds. HCV recruits specific components of SGs to viral replication factories and these co-localise with the HCV core protein and likely interact with the viral RNA to mediate efficient replication (Ariumi et al., 2011; Yi et al., 2011).

1.12 miRNA and siRNA-mediated post-transcriptional RNA silencing pathways

Small RNAs that associate with the RNA-induced silencing complex (RISC) are approximately 20-30 nucleotides in length and consist of 5' phosphate and 3' OH groups. Most small RNAs are generated through the action of RNase III enzymes, specifically DICER, although piRNAs are derived from alternative DICER-independent mechanisms. Following biogenesis small RNAs are sorted and associate with specific AGO proteins and it is this significant process that determines their biological function [reviewed in (Czech and Hannon, 2011)]. AGO proteins are central components of the RISC and in humans consist of eight proteins that can be divided into two subfamilies: the PIWI subfamily: HIWI (human PIWI), HILI (HIWI-like), PIWIL3 (PIWI-like 3), and HIWI2, and the AGO subfamily: AGO1, AGO2, AGO3 and AGO4 (Sasaki et al., 2003). In mammals, expression of the PIWI subfamily is restricted to germ cells where they bind to piRNAs and mediate sequence-specific transcriptional gene silencing (TGS) through DNA methylation. Alternatively, the AGO protein subfamily associates predominantly with RNase III digested small RNAs, namely miRNAs and short-interfering RNAs (siRNAs) that are generated as RNA duplexes. During RISC assembly only the miRNA or siRNA guide strand is retained, whereas the passenger

strand is ejected (Matranga et al., 2005). The guide strand directs the RISC complex to a complementary RNA target that is either translationally repressed or cleaved (Figure 1.9).

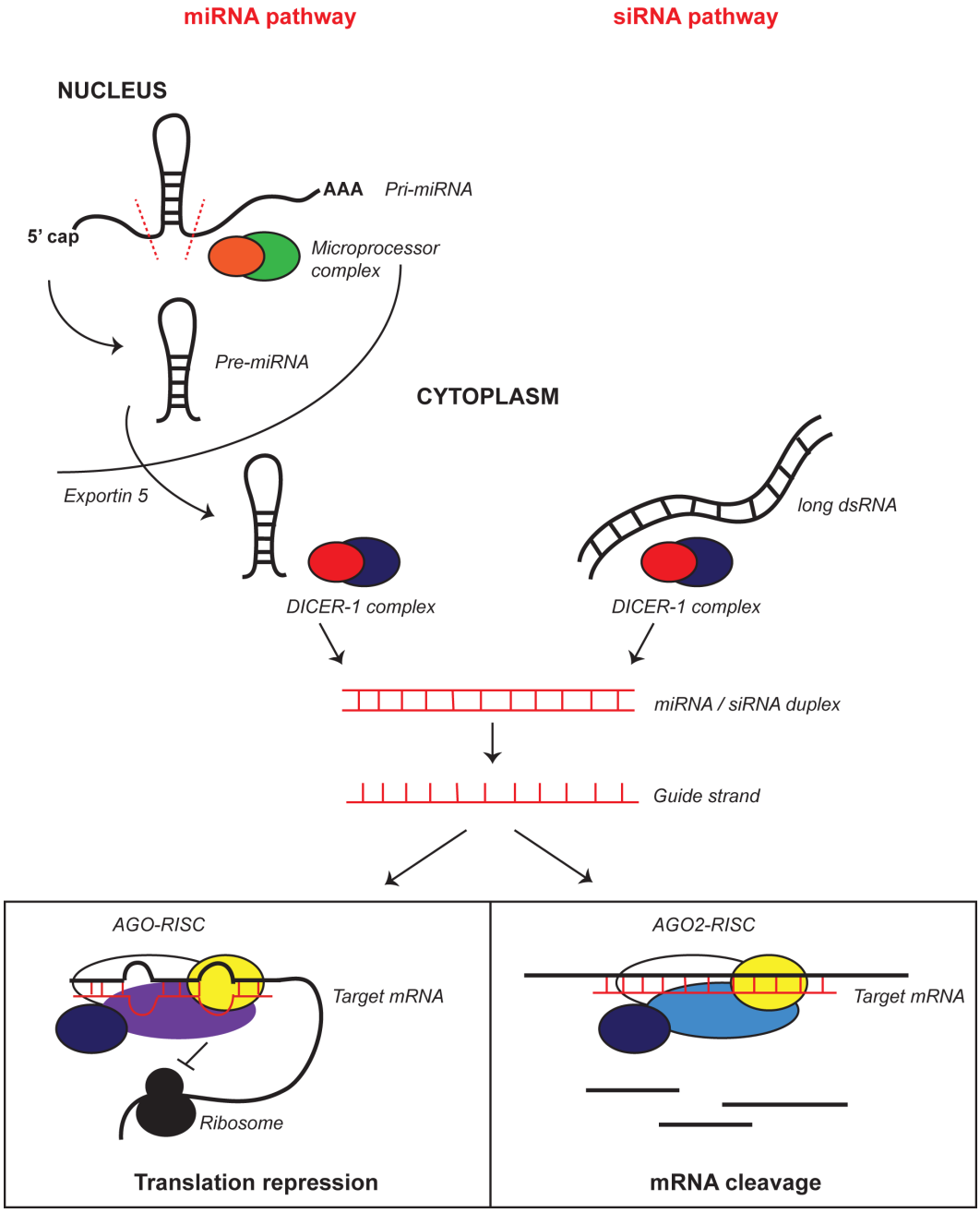


Figure 1.9. miRNA and siRNA-mediated RNA silencing pathways.

miRNAs are genome-encoded small RNAs that are processed in the nucleus from capped and polyadenylated primary miRNAs (pri-miRNAs) into precursor miRNAs (pre-miRNAs) by the microprocessor complex and exported into the cytoplasm by Exportin 5. Further processing of the pre-miRNA by the DICER-1 complex in the cytoplasm generates a miRNA: miRNA duplex. Similarly, the DICER-1 complex cleaves long double-stranded RNA precursors into siRNA: siRNA duplexes as well. The passenger strand is ejected from the duplex and the guide strand is loaded onto an Argonaute (AGO)-RNA-induced silencing complex (RISC). Imperfect complementarity between the guide strand and target mRNA results in translation repression (left box) and perfect complementarity results in cleavage of the target mRNA (right box). Only the AGO2 protein possesses slicer activity and, therefore, mediates mRNA cleavage.

1.12.1 miRNA biogenesis

miRNAs are ubiquitously encoded in the human genome and are transcribed by RNA pol II generating a primary miRNA (pri-miRNA) with a stem-loop structure that contains the mature miRNA (Lee et al., 2004). Analogous to protein-coding transcripts, the pri-miRNA is also 5' capped and polyadenylated (Lee et al., 2004). The double-stranded RBP Pasha/DiGeorge syndrome critical region gene 8 (DGCR8) and the RNase III enzyme Drosha form a complex called the Microprocessor that processes the pri-miRNA into an approximately 60-70 nucleotide precursor miRNA (pre-miRNA) (Denli et al., 2004; Gregory et al., 2004). RNase III-mediated digestion results in a characteristic 2 nucleotide single-stranded 3' overhang, which is recognised by Exportin 5 that orchestrates the Ran-GTP-dependent nuclear export of the pre-miRNA (Lund et al., 2004). Together with its double-stranded RBP partner TAR RNA-binding protein 2 (TARBP2), DICER-1 mediates the cleavage of the pre-miRNA into an approximately 22-23 nucleotide miRNA: miRNA duplex (Bernstein et al., 2001; Chendrimada et al., 2005; Haase et al., 2005).

1.12.2 siRNA biogenesis

These small RNAs were initially identified in plants (Hamilton and Baulcombe, 1999), and were shown to be derived from double-stranded RNAs generated either as intermediates of viral replication or through the activity of an RNA-dependent RNA polymerase (RdRP) (Fire et al., 1998). Studies in *Drosophila melanogaster* have shown that the introduction of long double-stranded RNAs results in DICER-2-mediated processing into siRNAs that are approximately 21 nucleotides in length (Lee et al., 2004). *Drosophila melanogaster* also encode endo-siRNAs that are expressed from endogenous loci and are likely involved in retrotransposon silencing. Endo-siRNAs can originate from RNA transcripts with hairpin structures, transposon clusters and also through the annealing of sense and antisense transposon transcripts (Czech et al., 2008; Ghildiyal et al., 2008; Okamura et al., 2008). Interestingly, an RdRP has also been in identified in *Drosophila melanogaster* (Lipardi and Paterson, 2009). Furthermore, endo-siRNAs have been identified in mouse oocytes and embryonic stem cells (Tam et al., 2008; Watanabe et al., 2008). As with exogenous siRNAs, the production of endo-siRNAs is dependent on DICER activity. In contrast, endo-siRNAs in plants and *Caenorhabditis elegans* are generated by a RdRP that copies single-stranded RNAs into long double stranded RNAs that may be processed by DICER activity.

1.12.3 Argonaute (AGO) proteins

The AGO protein family is well conserved in various species with one member in *Schizosaccharomyces pombe*, five in *Drosophila melanogaster*, eight in humans, ten in *Arabidopsis thaliana*, and twenty-seven in *Caenorhabditis elegans* [reviewed in (Ender and Meister, 2010)]. In humans, the AGO subfamily is ubiquitously expressed in somatic cells, whereas the PIWI subfamily is restricted to the germ line. In terms of functional domains, the AGO proteins are characterised by a PIWI-Argonaute-Zwille (PAZ) domain that binds to single-stranded RNA dinucleotide overhangs akin to those generated by DICER processing, and a PIWI domain similar to RNaseH, containing the catalytic activity (Cerutti et al., 2000). Both siRNAs and miRNAs bind to members of the AGO subfamily of proteins, whereas piRNAs associate with the PIWI proteins. These small RNAs guide AGO-containing RISCs to target mRNAs for translation silencing. Of the four human AGO proteins, only AGO2 mediates the endonucleolytic cleavage of perfectly complementary target mRNAs (Liu et al., 2004; Meister et al., 2004). In addition all AGO proteins can also silence target mRNAs with incomplete complementarity through translation repression and the recruitment of mRNA decapping and deadenylation machinery, likely through GW182 (Pillai et al., 2004; Sen and Blau, 2005; Behm-Ansmant et al., 2006). However, the exact mechanisms underlying miRNA-mediated translation repression are still unclear.

1.12.4 RISC assembly and silencing

Mammals harbour a single DICER enzyme that sorts miRNAs and siRNAs between four AGO subfamily proteins. Studies in *Drosophila melanogaster*, in which two DICER enzymes preferentially load miRNAs into AGO1 complexes for translation repression, and siRNAs into AGO2 complexes for slicing, show that the characteristic of the RNA duplex precursor may be important for the loading bias. Precursor siRNA duplexes are highly or even perfectly complementary and, on the other hand, miRNA duplexes demonstrate imperfect base pairing, mismatches and bulges. Secondly, terminal nucleotides also contribute to the sorting decision, whereby AGO1 prefers a terminal U and AGO2 favours a 5' C (Czech et al., 2009). Interestingly, the human AGO clade of proteins seem to portray no preference for duplex structure or terminal nucleotide, implying a more relaxed system of sorting (Liu et al., 2004; Meister et al., 2004).

Loading of the RISC with miRNAs and siRNAs is an ATP-dependent process that requires assistance from a number of proteins (Yoda et al., 2010). AGO proteins accept RNA duplexes following which the passenger strand is discarded during RISC maturation. Both mammalian and *Drosophila melanogaster* AGO2 proteins can cleave the passenger strand in AGO2 complexes leading to maturation of the AGO2-RISC (Matranga et al., 2005). This process is less well understood for AGO1, AGO3 and AGO4 complexes, although the mechanism has been proposed to be cleavage-independent (Kawamata et al., 2009). The guide strand directs the mature RISC to a target mRNA based on the complementarity of the seed region (2^{nd} to 8^{th} nucleotide) with the 3' UTR of the target resulting in miRNA and siRNA-mediated translation repression or mRNA cleavage; although additional extensive base-pairing outside the seed region is also necessary for target mRNA cleavage.

1.12.5 miRNA and siRNA-mediated regulation of retroelements

RNA silencing is a key regulatory mechanism in eukaryotic cells with roles in various cellular processes, such as cellular development, differentiation, cell death as well as protection against genetic parasites. As exogenous and endogenous retroelements depend on the host cell for efficient replication, their interaction with components of the RNA silencing pathway is not unanticipated. HIV-1 infection has been reported to induce the accumulation of virus-specific small RNAs and, particularly, an 18 nucleotide molecule antisense to the HIV-1 PBS, which is generated in a DICER-1-dependent manner and shown to associate with AGO2 (Yeung et al., 2009). Antiviral RNA interference (RNAi) has been a controversial concept in mammals, particularly owing to the lack of an RdRP for the production of RNA duplexes; however, the recent discovery of an RdRP in humans formed by an interaction between the human telomerase RT catalytic subunit (TERT) and the RNA component of mitochondrial RNA processing endoribonuclease (RMRP) (Maida et al., 2009), justifies careful examination of this regulatory pathway as a potential antiviral mechanism.

Cellular miRNAs have also been implicated in the negative regulation of HIV-1 replication and in the induction of latency. In resting CD4+ T cells, miR-28, miR-125b, miR-150, miR-223 and miR-382 have been reported to target sequences located at the 3' end of viral transcripts resulting in the suppression of their expression (Huang et al., 2007). In contrast, the abundance of these miRNAs is reduced in activated CD4+ T

cells, which is thought to permit HIV-1 replication (Huang et al., 2007). Similarly, miR-28, miR-150, miR-223, and miR-382 have been proposed to contribute to the resistance of peripheral blood monocytes to HIV-1 infection, whereby the suppression or induction of these miRNAs in monocytes has been suggested to facilitate or inhibit infectivity, respectively (Wang et al., 2009). Likewise, the miR-29a was reported to target a sequence within the HIV-1 *nef* gene, restricting Nef expression and HIV-1 replication, and these interactions were suggested to increase the association of HIV-1 genomic RNA with RISC and P bodies (Ahluwalia et al., 2008; Nathans et al., 2009). Furthermore, depletion of DICER-1 has been suggested to enhance both HIV-1 virus production and infectivity (Nathans et al., 2009). Virus encoded suppressors of RNA silencing (SRS) imply that viral replication may be restricted by miRNAs or siRNAs. Taking this into account, the HIV-1 Tat protein has been proposed to possess SRS activity through mutagenesis and heterogeneous chimera studies with SRS factors from plant viruses (Bennasser et al., 2005; Schnettler et al., 2009).

Analogous to the negative regulation of HIV-1 by cellular miRNAs, the LINE-1 retrotransposon contains a sense and antisense promoter in the 5' UTR, and bidirectional transcription from these promoters has been reported to generate LINE-1 encoded endo-siRNAs that can restrict LINE-1 retrotransposition by RNAi (Yang and Kazazian, 2006). Consistently, a higher abundance of LINE-1 and IAP transcripts are detected in *Dicer-1* knockout mouse embryonic stem cells (Kanellopoulou et al., 2005). Naturally occurring endo-siRNAs have also been reported to negatively regulate human LINE-1 expression, although the resultant mechanism for suppression is DNA methylation of the LINE-1 promoter (Chen et al., 2012).

1.12.6 miRNA and siRNA-mediated regulation of other RNA viruses

Although cellular miRNAs have been implicated in the inhibition of HIV-1 replication, not all cellular miRNAs negatively regulate RNA viruses. The miR-122, which is expressed at high levels in human liver cells, is required for HCV replication, and directly binds to two target sites in the 5' noncoding region of the viral genome regulating gene expression (Jopling et al., 2005; Jopling et al., 2008). Inhibitors of miR-122 are currently being investigated as potential therapies for treating HCV infection. (Lanford et al., 2010)[reviewed in (Jopling, 2010)]. On the contrary, induction of an IFNβ response during HCV replication was reported to upregulate the

anti-HCV cellular miRNAs miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431 and miR-448 and, in sync with previous reports, was suggested to downregulate expression of the HCV cofactor miR-122 (Pedersen et al., 2007).

1.13 Moloney leukemia virus 10 (MOV10)

Gallois-Montbrun et al identified the putative RNA helicase MOV10 as a A3F and A3G-interacting RBP (Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008) (section 1.9). RNA helicases use ATP to remodel RNA or RNP complexes and can be classified into superfamilies (SFs) based on sequence similarity, structure and other mechanistic features. The helicase core can contain up to 13 sequence motifs that carry out ATP binding and hydrolysis, nucleic acid binding and the coordination of these functions. Domains involved in binding and hydrolysis of ATP, such as the Walker A and Walker B motifs, are fairly conserved between SFs. MOV10 is a SF1 Upf1-like DEAG-box putative RNA helicase, whereby the 'DEAG-box' denotes the sequence of its Walker B motif [reviewed in (Fairman-Williams et al., 2010)] (Figure 1.10). The C-terminal domain of MOV10 consists of the putative RNA helicase motifs, however, the N-terminal domain contains no known protein sequences.

MOV10, also known as GB110 (GTP-binding 110kda protein) in the mouse, was originally identified through an MLV proviral integration into the GB110 locus in the *Mov-10* mouse strain (Mooslehner et al., 1991). Functional studies showed that mouse embryonic stems cells lacking functional GB110 proliferated and differentiated analogous to wild-type cells (Hamann et al., 1993). Human MOV10 is ubiquitously expressed in a range of adult tissues such as the heart, brain, lungs, liver, testes and ovaries with lower transcript levels detected in the smooth muscle, and highest transcript abundance in the adult CNS (Nagase et al., 2000). Interestingly, cancer cells express higher levels of both MOV10 mRNA and protein in comparison with healthy cells (Nakano et al., 2009).

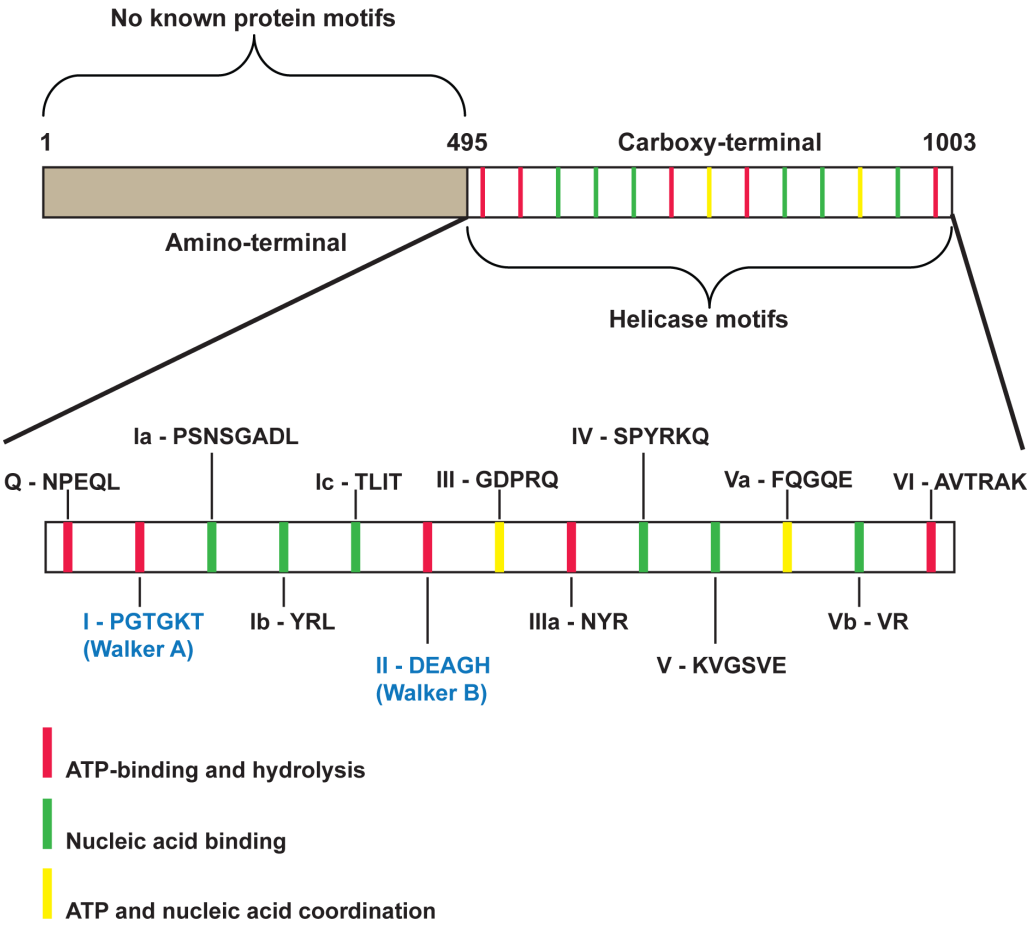


Figure 1.10. MOV10 domains and putative helicase motifs.

The amino (N)-terminal (residues 1-495) of MOV10 contains no known protein motifs and the carboxy (C)-terminal (residues 496-1003) contains potentially thirteen putative RNA helicase motifs. Five of these motifs have roles in ATP-binding and hydrolysis (illustrated in red), six function in nucleic acid binding (illustrated in green) and two coordinate ATP and nucleic-acid binding. The Walker A and Walker B putative helicase motifs, which are highly conserved domains required for ATP-binding and hydrolysis, are additionally highlighted in blue.

1.13.1 Cellular associations of MOV10

MOV10 interacts with the antiviral factors A3F and A3G in an RNA-dependent manner and also co-localises with these proteins in cytoplasmic mRNA P bodies and SGs (Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). The RNA-dependent interaction between MOV10 and A3G has been proposed to be bridged by the 7SL RNA (Liu et al., 2012); however, a recent 'interactome capture' study that determined the RNAs bound by a panel of RBPs demonstrated that MOV10 broadly associates with a range of RNAs as opposed to interacting with a distinct subset (Castello et al., 2012), implying that multiple RNAs may be responsible for this interaction.

Proteomics analyses and co-immunoprecipitation experiments have identified MOV10 as interacting with the AGO proteins, AGO1 and AGO2 (Meister et al., 2005; Chendrimada et al., 2007). These are essential constituents of the RISC, which is an RNP complex that directs small RNA-mediated post-transcriptional RNA silencing. MOV10 also associates with mature AGO complexes loaded with miRNAs (Meister et al., 2005). AGO1 and AGO2 co-localise with MOV10 in P bodies and, interestingly, AGO proteins also interact with A3G and A3F although in an RNA-independent manner unlike MOV10 (Meister et al., 2005; Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). Considering the association with AGO proteins and the RISC, MOV10 has been reported to be necessary for miRNA-guided cleavage of a reporter mRNA in cultured cells (Meister et al., 2005). In contrast, Qi et al could not detect an interaction between MOV10 and the AGO1 or AGO2 proteins, although they identified MOV10 as an abundant protein in AGO3 and AGO4 purifications by mass spectrometry (Qi et al., 2008). DICER-1 is an RNase III enzyme essential for the biogenesis of miRNAs and siRNAs (section 1.12), however, two groups reported conflicting data regarding the association of MOV10 with this enzyme reflecting a possible weak or transient association (Meister et al., 2005; Chendrimada et al., 2007)

Huang et al proposed the cellular function of members of the APOBEC3 family, including A3G, to be the derepression of miRNA-mediated translation repression (Huang et al., 2007) and, interestingly a recent study suggested that A3G mediates this activity by interfering with the AGO2-MOV10 interaction, and consequently preventing maturation of the RISC (Liu et al., 2012). However, Phalora et al recently ruled out a

role for APOBEC3 proteins in the specific regulation of miRNA function (Phalora et al., 2012).

The broad RNA-binding capacity of MOV10 (Castello et al., 2012) likely explains the detection of MOV10 in a number of screens searching for RBP interaction partners. MOV10 copurifies with the mouse Y RNA-binding Ro protein in complex with the zipcode-binding protein (ZBP1) and YB-1 (Sim et al., 2012). Ro has been implicated in cell survival following stress and the quality control of non-coding RNAs, both of which are modulated by Y RNAs (Sim and Wolin, 2011). ZBP1 functions in RNA metabolism pathways with roles in mRNA localization, stability and translation as well as implications in stress responses (Huttelmaier et al., 2005; Stohr et al., 2006), and similarly YB-1 is a translation repressor with functions in cancer biology as well as cellular stress (Evdokimova et al., 2006; Yang and Bloch, 2007). Interestingly, YB-1 also interacts with A3G and A3F in a partially RNase-sensitive manner (Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). MOV10 was also identified in Staufen2 complexes, with Upf1 and RNA helicase A (RHA) (Miki et al., 2011). Staufen2 is an RBP that regulates mRNA transport and translation in neurons (Duchaine et al., 2002) and its paralog Staufen1 localises to SGs where it has been suggested to function in recovery from cellular stress (Thomas et al., 2009). Staufen1 has also been suggested to play a role in Upf1-mediated mRNA decay (Kim et al., 2005). Furthermore, RHA has been implicated in the translation of specific mRNAs, including HIV-1 transcripts and has also been identified as a RISC component (Hartman et al., 2006; Robb and Rana, 2007). The functional relevance of these interactions remains to be determined.

Consistent with a potential role in post-transcriptional translation regulation, studies using rat hippocampal neurons have implicated MOV10 in a local translation control mechanism contributing to synaptic plasticity, whereby proteasomal degradation of MOV10 at synapses allows the association of specific mRNAs with polysomes for protein synthesis (Banerjee et al., 2009). Similarly, MOV10 is also degraded by the proteasome at synapses in the rat amygdala relieving translational silencing and permitting long-term memory formation and retrieval (Jarome et al., 2011). Besides post-transcriptional control of translation, Messaoudi-Aubert et al identified human MOV10 as interacting with the Polycomb-repressive complex 1 (PRC1), which consists

of the Polycomb group (PcG) proteins that function in noncoding RNA-mediated transcriptional repression. Silencing of endogenous MOV10 was reported to upregulate the *INK4a* tumour suppressor gene accompanied by a reduction in histone methylation at this target locus (Messaoudi-Aubert et al., 2010).

1.13.2 MOV10 homologs

Post-transcriptional gene silencing (PTGS), later termed RNAi, is a mechanism by which eukaryotes such as higher plants, *Caenorhabditis elegans* and *Drosophila melanogaster* repress viruses and transposons at the RNA level (Ratcliff et al., 1997; Jensen et al., 1999; Ketting et al., 1999). The RNAi process is initiated with a long double-stranded RNA product derived from the genetic element by a number of mechanisms, including the activity of an RdRP in plants (Fire et al., 1998) (section 1.12.2). The precursor double-stranded RNA is cleaved into small 21-23 nucleotide RNAs, which are loaded onto a RISC that mediates cleavage of a complementary RNA species (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000) (section 1.12). In *Arabidopsis thaliana,* the silencing defective protein 1 (SDE1) is an RdRP and one of four silencing defective locuses. The silencing defective protein 3 (SDE3) is a MOV10 ortholog and, similar to SDE1, has been shown to be required for PTGS of the cucumber mosaic virus and a green fluorescent protein (GFP) transgene (Dalmay et al., 2001). More recently, Garcia et al revealed SDE3 RNA-helicase activity and its AGO binding ability to be important for this antiviral action, as well as for transposon silencing via a pathway correlative with DNA methylation (Garcia et al., 2012).

In *Drosophila melanogaster*, RNAi against retrotransposons is accomplished by the PIWI subfamily of the AGO proteins, which includes the PIWI, Aubergine (Aub) and AGO3 proteins. These proteins interact with and are guided by small RNAs called piRNAs that are derived in a DICER-independent manner mainly from piRNA clusters, composed of a dense population of truncated and immobilised sense and antisense orientation transposons (Brennecke et al., 2007). Aub and AGO3 bound piRNAs overlap at the 5'ends by exactly 10 nucleotides, leading to the proposal of a model whereby Aub and AGO3 generate piRNAs by a slicer activity-dependent amplification loop termed the ping-pong cycle (Gunawardane et al., 2007). Alternatively, the PIWI protein is a recipient of mainly cluster-derived piRNAs. A report has determined that

all three members of the PIWI subfamily function in the germline, however, the PIWI protein alone also plays a role in somatic cells, whereby it interacts with piRNAs derived from the *flamenco* cluster to restrict particular families of retrotransposons (Malone et al., 2009). Interestingly the MOV10 ortholog, Armitage, in *Drosophila melanogaster* is necessary for PIWI protein function, and knockdown of Armitage was shown to de-silence a retrotransposon reporter construct regulated by the *flamenco* cluster (Malone et al., 2009; Olivieri et al., 2010). The PIWI protein co-immunoprecipitates with Armitage, and silencing of endogenous Armitage decreases the levels and nuclear localisation of PIWI in both the germ line and somatic cells (Olivieri et al., 2010). Furthermore, evidence suggests that Armitage functions in assembly and maturation of the RISC, whereby mutations in Armitage inhibit RNAi (Tomari et al., 2004). Armitage likely plays a role in loading and assemby of the PIWI complex allowing its translocation into the nucleus, a process thought to be necessary for PIWI protein function (Saito et al., 2010).

The mouse PIWI proteins MIWI (mouse PIWI), MILI (Miwi-like) and MIWI2 are expressed specifically in the mouse germ line. Similar to the PIWI proteins in *Drosophila melanogaster* these proteins associate with piRNAs, however, in mice piRNAs are derived from discrete transposons as opposed to clusters, and there is also evidence for a MILI-dependent amplification loop (Aravin et al., 2007). PIWI proteins in mammals control retrotransposons by TGS established by the methylation of these endogenous retroelements (Kuramochi-Miyagawa et al., 2008). MOV10-like 1 (MOV10L1) is a germ cell-specific mammalian paralog of MOV10 that interacts with MIWI, MILI and MIWI2 (Frost et al., 2010; Zheng et al., 2010). Depletion of MOV10L1 decreases the abundance of MIWI and MILI proteins ultimately resulting in DNA demethylation and, therefore, derepression of LINE-1 and IAP endogenous retroelements (Frost et al., 2010; Zheng et al., 2010). MOV10L1 mutant male mice are sterile due to the apoptosis of spermatocytes, which correlates with an upregulation in retrotransposon transcripts and proteins (Frost et al., 2010). Interestingly, the expression of MOV10L1 has been reported to be impaired in Cryptorchid boys at high risk of azoospermia conferring clinical relevance to MOV10L1 in male infertility (Hadziselimovic et al., 2011).

1.13.3 MOV10 and RNA viruses

MOV10 has been implicated in the regulation of several RNA viruses suggesting that the capacity of MOV10 to regulate genetic elements has been evolutionarily conserved. Hepatitis delta virus (HDV) is a small RNA virus that encodes only the hepatitis delta antigen (HDAg) and, therefore, relies heavily on host factors for RNA-directed transcription of its genome. MOV10 has been identified as an HDAg-interacting protein that also associates with a species of HDV-derived small 5' capped RNAs implicated in viral transcription initiation (Haussecker et al., 2008). Furthermore, knockdown of endogenous MOV10 was shown to have no effect on HDAg translation, however, HDV replication was inhibited suggesting that MOV10 may play a role in HDV RNA-directed transcription (Haussecker et al., 2008). In contrast, MOV10 overexpression inhibits HCV infection and, similarly, silencing of endogenous MOV10 enhances the replication of vesicular stomatitis virus (VSV), the mechanisms for which remain to be established (Li et al., 2011; Schoggins et al., 2011). A number of groups have now also reported that MOV10 overexpression potently inhibits HIV-1, SIV and MLV infectivity amongst other retroviruses, although the target/s and molecular mechanism/s for this inhibition still require further investigation. Furthermore, the effect of silencing endogenous MOV10 on HIV-1 infectivity remains unclear, and has not yet been tested for other retroviruses (Burdick et al., 2010; Furtak et al., 2010; Wang et al., 2010). The findings of these studies will be discussed in greater detail in later chapters.

1.14 Aims

The identification of host cell factors that can negatively (restriction factors) or positively (cofactors) regulate retroelements is imperative to understand the capacity of the host to protect its genome from the potentially detrimental effects of retroviruses and retrotransposons. Several genome-wide screens have now identified thousands of cellular factors and multiple pathways that can stimulate or suppress HIV-1 infection, however, only thirty-four of these genes have been identified in two or more studies and, furthermore, a number of known and confirmed cellular factors are absent from these screens (Brass et al., 2008; Konig et al., 2008; Zhou et al., 2008; Liu et al., 2011). Therefore, the search for host restriction factors and cofactors that can modulate HIV-1 infection is very much an active area of research. Furthermore, with their associations in cancer biology as well as non-hereditary neurological diversity and disease, the discovery of factors that can suppress retrotransposons is similarly crucial.

MOV10 is a putative RNA helicase that controls the replication of several RNA viruses and whose homologs function in the restriction of viruses and endogenous retroelements. MOV10 interacts with members of the APOBEC3 family that restrict the replication of both retroviruses and retrotransposons, as well as the AGO proteins, which are core constituents of the RISC. Furthermore, MOV10 co-localises with A3F, A3G and the AGO proteins in P bodies and SGs, which are cytoplasmic sites involved in mRNA storage and decay. P bodies, SGs and miRNA or siRNA-mediated post-transcriptional RNA silencing pathways have been implicated in the regulation of retroelements as well as other positive-strand RNA viruses. Considering these associations of MOV10, this thesis will broadly aim to investigate the role of MOV10 in the replication of exogenous retroviruses from the lentivirus (HIV-1, HIV-2 and SIVmac), gammaretrovirus (MLV) and betaretrovirus (M-PMV) subfamilies. The effect of MOV10 on the retrotransposition of the mouse LTR-containing ERV IAP, and the human non-LTR LINE-1 and Alu retrotransposons will also be determined. Furthermore, mechanistic and structure-function studies will begin to dissect the targets and functions of MOV10 in the retroelement life cycle, as well as aim to better understand the cellular function of MOV10 and its relationship with the APOBEC3 proteins. Overall this thesis will analyse the potential of MOV10 as a novel restriction factor, or cofactor for retroviruses and retrotransposons.

CHAPTER 2
MATERIALS AND METHODS

2.1 Plasmids

The pCMV4-MOV10-HA plasmid was constructed by cloning a HindIII-XbaI digested MOV10 PCR product into the pCMV4 expression plasmid containing three 3' HA epitope tags. The pT7-MOV10 plasmid was constructed by cloning an XbaI-BamHI digested MOV10 PCR product into the pCGTHCFFLT7 (pT7) expression vector that contains two 5' T7-epitope tags (Caceres et al., 1998). The pcDNA3.1-myc-TIA-1 plasmid was generated by cloning an EcoRI-XhoI digested TIA-1 PCR product into the pcDNA3.1(+) plasmid that contains three 5' myc epitope tags. The N-terminal MOV10 mutant plasmid pT7-MOV10-N was constructed by cloning an XbaI-BamHI digested N-terminal MOV10 PCR fragment (residues 1-495) into the pT7 expression vector. Similarly, the C-terminal MOV10 mutant plasmid pT7-MOV10-C was constructed by cloning an XbaI-BamHI digested C-terminal MOV10 PCR product (residues 496-1003) into the pT7 plasmid. To construct the MOV10 putative helicase mutant plasmids pT7-MOV10-K530A, pT7-MOV10-DE645AA and pT7-MOV10-G648A, full-length MOV10 PCR products containing the relevant mutations were generated by overlapping polymerase chain reaction (PCR) and NheI-XmaI digested products were cloned into a similarly digested pT7-MOV10 backbone. The shRNA-resistant pT7-MOV10-R plasmid was generated by producing a full-length MOV10 PCR product containing six silent mutations in the MOV10-specific shRNA target sequence (nucleotides 342 to 363) by overlapping PCR and cloning the XbaI-XmaI digested product into a similarly digested pT7-MOV10 plasmid.

The pcDNA3.1-myc-MOV10 and pcDNA3.1-myc-GFP control plasmids were generated by Dr. Sarah Gallois-Montbrun, the pCMV4-GFP-HA control plasmid was generated by Dr. Prabhjeet Phalora, and Dr. Chad Swanson cloned the pT7-GFP and pT7-Luc control plasmids. Additionally, The subgenomic GPV-RRE construct was generated by Dr. Nathan Sherer by insertion of a SacI-EcoRI digested fragment of the pHIV-1_{NL4-3} plasmid into the pcDNA3.1(+) plasmid consisting of the RRE and CMV promoter-5'LTR fusion (Cullen, 1986). The ΔMA+M-src and ΔNC+Z_t GPV-RRE

mutant constructs were also generated by Dr. Nathan Sherer by cloning SacI-AgeI digested fragments from pHIV-1_{NL4-3} (ΔMA+M-src) and pHIV-1_{NL4-3} (ΔNC+Z_t), which were kind gifts from Dr. David Ott, into a similarly digested GPV-RRE construct, and the codon-optimised GPV-RRE construct was also cloned by Dr. Nathan Sherer. The pRev plasmid was generated by Dr. Chad. Swanson by cloning the Rev cDNA into the pcDNA3.1(+) plasmid.

The pcDNA3.1-myc-AGO2 plasmid was a kind gift from Dr. Gregory Hannon. The pHIV-1_{NL4-3} proviral plasmid was a kind gift from Dr. Malcolm Martin (Adachi et al., 1986). Plasmids for SIVmac vector production, pSIV3+ and pSIV-RMES4, were kind gifts from Dr. François-Loïc Cosset (Negre et al., 2000), and plasmids for HIV-1 vector production, p8.91 and pCSGW, were kind gifts from Dr. Didier Trono and Dr. Robin Ali, respectively (Zufferey et al., 1997; Bainbridge et al., 2001). Plasmids for MLV virus production, pMLV and pMLV-Tat, were kind gifts from Dr. Stephen Goff and Dr. Juan Martin-Serrano, respectively (Martin-Serrano et al., 2003; Yueh and Goff, 2003). The plasmid for M-PMV virion production, pMTΔE, was a kind gift from Dr. Brian Cullen (Doehle et al., 2006). The pVSV-G plasmid has been described previously (Fouchier et al., 1998). The full-length pSIVmac239 and pHIV-2_{ROD} proviral plasmids were kind gifts from Dr. Stuart Neil. For endogenous retroelement experiments, the pGL3-IAP92L23neo^{TNF}, pAlu-neo^{Tet} and pORF2p plasmids were kind gifts from Dr. Thierry Heidmann (Dewannieux et al., 2003; Dewannieux et al., 2004), and the pJM101/L1.3 plasmid was a kind gift from Dr. John Moran (Moran et al., 1999). For Luc assays, the firefly Luc reporter constructs were kind gifts from Dr. Joan Steitz (Lytle et al., 2007), and the renilla Luc reporter constructs were kind gifts from Dr. Gyorgy Hutvagner (Johnston et al., 2010). The pHIV-1_{IIIB}/ΔVif, pCMV4-A3A-HA, pCMV4-A3B-HA and pCMV4-A3G-HA plasmids have been described previously (Bishop et al., 2004).

Expression plasmids were verified by restriction enzyme mapping and/or sequencing where appropriate. The primers used for cloning are listed in table 2.1.

Table 2.1. Primers for cloning.

Plasmid	Primer name	Restriction site	Sequence
pCMV4-MOV10-HA	oSA15	HindIII (Fwd)	AAAAAGCTTATGCCCAGTAAGTTCAGC
pCMV4-MOV10-HA	oSA16	XbaI (Rev)	AAATCTAGAGAGCTCATTCCTCCACTC
pT7-MOV10	oSA17	XbaI (Fwd)	AAATCTAGACCCAGTAAGTTCAGCTGCCGG
pT7-MOV10	oSA18	BamHI (Rev)	AAAGGATCCTCAGAGCTCATTCCTCCA
pcDNA3.1-myc-TIA-1	oSA3	EcoRI (Fwd)	CCGGAATTCATGGAGGACGAGATGCCCA
pcDNA3.1-myc-TIA-1	oSA4	XhoI (Rev)	GCCTCGAGTTCACTGGGTTTCATACCCTGC
pT7-MOV10-N	oSA17	XbaI (Fwd)	AAATCTAGACCCAGTAAGTTCAGCTGCCGG
pT7-MOV10-N	oSA49	BamHI (Rev)	AAAGGATCCTCACCGGTCGTACAGCTT
pT7-MOV10-C	oSA50	XbaI (Fwd)	AAATCTAGAAGTCTGGAGTCAAAC
pT7-MOV10-C	oSA18	BamHI (Rev)	AAAGGATCCTCAGAGCTCATTCCTCCA
pT7-MOV10-K530A	oSA64	Overlapping (Fwd)	CCTCCAGGCACCGGCGCCACTGTCACGTTAGTG
pT7-MOV10-K530A	oSA72	Overlapping (Rev)	TTGCCTCCACTAACGTGACAGTGGCGCCGGTGCCTGGAGG
pT7-MOV10-K530A	oSA84	NheI (Fwd)	CCACAAGTCACTGCTAGCCAAGATC

pT7-MOV10-K530A	oSA85	XmaI (Rev)	GGGCCTTGGCCCGG GTCACAGCTAC
pT7-MOV10-DE645AA	oSA66	Overlapping (Fwd)	ACACACATCTTCAT CGCCGCGGCTGGCC ACTGCATG
pT7-MOV10-DE645AA	oSA73	Overlapping (Rev)	AGGCTCCATGCAGT GGCCAGCCGCGGCG ATGAAGATGTGTGT
pT7-MOV10-DE645AA	oSA84	NheI (Fwd)	CCACAAGTCACTGC TAGCCAAGATC
pT7-MOV10-DE645AA	oSA85	XmaI (Rev)	GGGCCTTGGCCCGG GTCACAGCTAC
pT7-MOV10-G648A	oSA68	Overlapping (Fwd)	TTCATCGATGAGGC TGCCCACTGCATGG AGCCT
pT7-MOV10-G648A	oSA74	Overlapping (Rev)	CCAGACTCTCAGGC TCCATGCAGTGGGC AGCCTCATCGATGA A
pT7-MOV10-G648A	oSA84	NheI (Fwd)	CCACAAGTCACTGC TAGCCAAGATC
pT7-MOV10-G648A	oSA85	XmaI (Rev)	GGGCCTTGGCCCGG GTCACAGCTAC
pT7-MOV10-R	oSA91	Overlapping (Fwd)	TTTATGACAGGGCC GAATACCTCCACGG AAAACATGGTGTGG
pT7-MOV10-R	oSA92	Overlapping (Rev)	CCACACCATGTTTTC CGTGGAGGTATTCG GCCCTGTCATAAA
pT7-MOV10-R	oSA17	XbaI (Fwd)	AAATCTAGACCCAG TAAGTTCAGCTGCC GG
pT7-MOV10-R	oSA85	XmaI (Rev)	GGGCCTTGGCCCGG GTCACAGCTAC

2.2 Reagents for RNAi

GIPZ lentiviral vectors encoding the non-silencing control and MOV10-specific shRNAs were purchased from Open Biosystems (V2LHS_201304). The non-silencing control and DICER-1 specific siRNAs were obtained from Ambion Life Technologies (s23756) and the efficiency of DICER-1 KD was measured by qPCR (section 2.12) with a TaqMan gene expression assay (Hs00229023_m1, Ambion Life Technologies). The shRNA and siRNA sense sequences are listed in table 2.2.

Table 2.2. shRNA and siRNA sense sequences.

Target	Sequence
MOV10	CTGAGTATCTTCATGGGAA
shRNA non-silencing control	ATCTCGCTTGGGCGAGAGTAAG
DICER-1	GGCUUAUAUCAGUAGCAAUTT

2.3 Cloning

2.3.1 Polymerase chain reaction (PCR)

Reactions were carried out in a total volume of 20.5 µl. The mixtures contained 0.5 µl of plasmid DNA as the template (100 ng/µl), 0.7 µl of the forward and reverse primer each (10 pmol/µl) (MWG Eurofins), 4 µl of F-518 5X Phusion HF buffer (provides approximately 1.5mM $MgCl_2$ in final reaction mixture) (Finnzymes, New England Biolabs (NEB)), 0.4 µl dNTP mix (10 mM each of dATP, dCTP, dGTP and dTTP), 0.2 µl of F-530L Phusion high-fidelity DNA polymerase (2 U/µl) (Finnzymes, NEB) and 14 µl of ddH_2O. PCR reactions were performed using the MJ Research PTC-200 Peltier Thermal Cycler with standard PCR conditions outlined in table 2.3. The PCR product (2 µl) was analysed by agarose gel electrophoresis (section 2.3.3) and purified using the QIAquick PCR purification kit (QIAgen) (section 2.3.4) prior to further steps.

Table 2.3. Standard PCR reaction conditions.

	Temperature	Time	Step
1	98° C	30 seconds	Initial denaturation
2	98° C	30 seconds	Denaturation
3	55° C	60 seconds	Annealing
4	72° C	15 – 60 seconds*	Extension
5	Go to step 2, repeat 30 times		
6	72° C	10 minutes	Final extension
7	4° C	Hold	

* Generally 15-30 seconds/kb, not exceeding 1 minute/kb.

2.3.2 Site-directed mutagenesis (overlapping PCR)

The first round of PCR included two PCR reactions, the end products of which consisted of the desired mutation/s: (1) Using an outer forward primer with an internal reverse primer containing the mutation (2) Using an internal forward primer containing the mutation with an outer reverse primer. The sequences of the forward and reverse internal primers should overlap. The PCR products from these reactions were separated on an agarose gel (section 2.3.3) and purified using the QIAquick gel extraction kit (QIAgen) (section 2.3.7). The PCR products were combined to form the template for the second round of PCR using the outer forward and reverse primers. The PCR reaction was performed as presented in table 2.3. The final PCR product was analysed by agarose gel electrophoresis (section 2.3.3) and purified using the QIAquick PCR purification kit (QIAgen) (section 2.3.4).

2.3.3 Agarose gel electrophoresis

1% agarose gels were prepared by adding 1 g of powdered agarose (Invitrogen) to 100 ml of TBE buffer (0.09 M Tris, 0.09 M borate, 2 mM EDTA, pH 8.4 (1X solution)) (Fisher Scientific) and bringing to a boil to dissolve the agarose powder. Following cooling of the solution, ethidium bromide was added (final concentration 0.5 μg/ml) and the mixture was poured into an electrophoresis tank to set. DNA samples were prepared

by mixing with 6X loading dye (2.5 % Ficoll 400, 11 mM EDTA, 3.3 mM Tris-HCl, 0.017 % SDS and 0.015 % Bromophenol Blue, pH 8.0) (NEB). Samples were loaded onto the set gel and run in 1X TBE buffer. The λ DNA-HindIII digest (band sizes 23130, 9146, 6557, 4361, 2322 and 2027bps) and ΦX174 DNA-HaeIII digest (band sizes 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72bps) DNA ladders were also run as markers to determine the DNA size. The gels were run at 100 volts for approximately 1hr. DNA bands were visualized on an ultraviolet (UV) trans-illuminator with a CCD camera and gel quantification software (Bio-Rad).

2.3.4 PCR product purification

Following analysis of the PCR product by agarose gel electrophoresis, the product was purified using the QIAquick PCR purification kit (QIAgen). For this, 100 µl of Buffer PB (contains guanidine hydrochloride) was added to approximately 18.5 µl of the remaining PCR reaction mixture and thoroughly mixed. The mixture was applied to a QIAquick spin column and centrifuged at 14000 rpm for 1 minute. The flow-through was discarded. To wash, 750 µl of Buffer PE (contains ethanol) was added to the column and centrifuged as described. The flow-through was again discarded and the column was centrifuged for a further 1 minute to remove any residual ethanol from Buffer PE. DNA was eluted in a sterile eppendorf tube by adding 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) to the column, leaving the column to stand for approximately 1 minute and then spinning as described.

2.3.5 DNA digestion by restriction endonucleases

Reactions were carried out in a total volume of 40 µl, which included 30 µl of the purified PCR product (or 2 µl of plasmid DNA (1 µg/µl) plus 28 µl of ddH_20), 1 µl of each restriction enzyme (NEB), 4 µl of the appropriate 10X buffer (listed in NEB catalogue) and 4 µl of bovine serum albumin (final concentration 100 µg/ml) (NEB). Digests were performed at 37° C for approximately 2-3hrs, unless the manufacturers instructions specified otherwise.

2.3.6 Dephosphorylation of digested DNA

Digested plasmid DNA was treated with 1 µl of calf intestinal alkaline phosphatase (CIP) (NEB) at 37° C for 1hr to prevent religation of compatible ends.

2.3.7 Gel extraction and purification

The digested DNA fragments were extracted and purified using the QIAquick gel extraction kit (QIAgen). For this the digested DNA reaction mixtures were separated on an agarose gel and visualised as described (section 2.3.3). Correct size DNA fragments were excised from the gel using a clean razor blade and transferred into sterile eppendorf tubes. To dissolve the gel slice, 300 µl of Buffer QG (contains guanidine thiocyanate) was added to the tubes and these were then incubated at 50° C for approximately 10 minutes, with some vortexing. 100 µl of isopropanol was added to the dissolved mixture and 700 µl of this was applied to a QIAquick spin column. The columns were centrifuged at 14000 rpm for 1 minute and the flow-through was discarded, and this step was repeated for any remaining dissolved gel mixture. 500 µl of Buffer QG was added to the columns and centrifuged as described, following which the columns were washed with 750 µl of Buffer PE and centrifuged as described. The flow-through was discarded and the columns were centrifuged for a further 1 minute to remove any residual ethanol from Buffer PE. The DNA was eluted into sterile eppendorf tubes by adding 50 µl of Buffer EB to the columns and leaving these to stand for approximately 1 minute prior to centrifugation as described. The yield of the digested plasmid and insert DNA was determined by running 5 µl of the elutions on an agarose gel (section 2.3.3).

2.3.8 DNA ligation

Reactions were carried out in a total volume of 10 µl, which generally included 7 µl of the digested insert, 1 µl of the digested plasmid, 1 µl of T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM Dithiothreitol, pH 7.5 (1X)) (NEB) and 1 µl of T4 DNA ligase (NEB). The ligation reaction was performed at room temperature for approximately 4hrs to overnight, and used to transform competent bacteria (section 2.4.3).

2.4 Bacteria

2.4.1 Bacterial strains and maintenance

Top10 *Escherichia coli* (E.coli) competent cells (Invitrogen) were used for the majority of transformations, except for the transformation of proviral DNA and lentiviral vectors for which Stable 2 (Stbl2) E.coli competent cells (Invitrogen) were used instead.

Bacterial cells were grown in Luria-Bertani (LB) Broth (1 LB tablet per 50 ml ddH_20; each tablet contains 10 g/L enzymatic digest of casein, 5 g/L yeast extract (low sodium), 5 g/L sodium chloride, 2 g/L inert agents, and 22 g/L total solids) (Sigma-Aldrich) or LB-Agar (37 g per 1 L of ddH_20) set in 10 cm sterile dishes (Sterilin) for solid phase growth cultures. Media was supplemented with the antibiotic Ampicillin (Calbiochem) to a final concentration of 100 μg/ml.

2.4.2 Production of competent bacteria

Top10 competent cells were inoculated into 10 ml of LB media overnight at 30°C with shaking at approximately 200 rpm. The next day 50 ml of LB was inoculated with 1.25 ml of the overnight bacterial culture and grown at 37°C with shaking as described until the optical density at 550 nm (OD550) reached between 0.45-0.55. The cells were harvested by centrifugation at 3500 rpm for 10 minutes at 4°C. The buffers for subsequent steps were pre-chilled on ice and filter sterilised. The bacterial cell pellet was resuspended in 20 ml of TfB1 buffer (30 mM KAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol and ddH_20 to a final volume of 100 ml) and chilled on ice for 5 minutes. The cells were centrifuged as described again and resuspended in 2 ml of TfB2 buffer (10 mM PIPES, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol and ddH2O to a final volume of 100 ml). The cell suspension was incubated on ice for 10 minutes and aliquoted into sterile eppendorf tubes, following which the competent bacterial cells were stored at -80°C.

2.4.3 Transformation of competent bacteria

Aliquots of competent bacterial cells were thawed on ice for approximately 10 minutes, and 30 μl of cells were incubated with 3 μl of the DNA ligation reaction or 1 μl of a plasmid stock (0.1 μg/μl) for 30 minutes on ice. Top10 bacterial cells were heat shocked at 42°C for 45 seconds and then recovered on ice for 2 minutes, following which 1 ml of sterile LB media was added to the cells and they were cultured at 37°C for 1hr. Stbl2 cells were transformed similarly, however, cells were heat shocked at 42°C for 30 seconds and cultured at 30°C for 90 minutes. For DNA ligation transformation reactions, bacterial cell cultures were centrifuged at 3500 rpm for 5 minutes and the pellets were resuspended in 100 μl of LB media before being plated onto LB-Agar plates containing Ampicillin. For plasmid stock transformations, 100 μl

of the bacterial culture was plated directly. Plates streaked with Top10 and Stbl2 bacterial cells were incubated overnight at 37°C or 30°C, respectively.

2.5 Plasmid DNA extraction and purification from bacterial cultures

2.5.1 Miniprep

A single colony was selected from a transformed plate of bacteria and inoculated in 2 ml of sterile LB media containing Ampicillin. These were incubated overnight at 37°C or 30°C as described with shaking and the next day 1 ml of the cultures was removed into sterile eppendorf tubes and centrifuged at 6000 g for 10 minutes to pellet the cells. The supernatant was aspirated and the pellets were resuspended in 100 µl Buffer P1 (50 mM Tris-HCL pH 8.0, 10 mM EDTA) containing RNase A (0.1 mg/ml final concentration) and then lysed in 100 µl Buffer P2 (200 mM NaOH and 1% SDS) by inverting the tubes 5X and incubating at room temperature for 5 minutes. Following this the lysed mixture was neutralized by adding 100 µl of Buffer P3 (3 M KOAc, pH 5.5) and again inverting the tubes 5X. The tubes were incubated on ice for 10 minutes and then centrifuged at 14000 rpm for 20 minutes. 250 µl of the supernatant was transferred into fresh sterile eppendorf tubes and 175 µl of isopropanol was added to precipitate the DNA. These were centrifuged as described and the supernatant was discarded. The clear DNA pellet was washed in 70% ethanol and centrifuged at 14000 rpm for 10 minutes. The supernatant was discarded and the DNA pellet was air dried to remove any traces of ethanol and then resuspended in 50 µl ddH_20.

2.5.2 Midiprep/Maxiprep

The midiprep/maxiprep was performed by alkaline lysis using the Machery-Nagel Nucleobond Xtra Midi/Maxi plasmid DNA purification kit (Machery-Nagel). A single colony was selected from a transformed plate of bacteria and inoculated in 50 ml/200 ml of sterile LB media containing Ampicillin. Bacterial cultures were incubated overnight at 37°C or 30°C as described with shaking. The next day, cultures were pelleted by centrifugation at 6000 g for 15 minutes and resuspended in 8 ml/12 ml of Buffer RES containing RNase A (0.06 mg/ml final concentration). The cells were lysed by adding 8 ml/12 ml of Buffer LYS, inverting 5X and incubating at room temperature for 5 minutes, following which 8 ml/12 ml of Buffer NEU was added and the lysate mixture was neutralised by gently inverting 15X. The precipitate was added to Nucleobond Xtra Columns that had been equilibrated with 12 ml/25 ml of Buffer EQU, and allowed

to empty by gravity flow. The columns were washed with 5 ml/15 ml of Buffer EQU, following which the column filter was discarded and the column was washed with 8 ml/25 ml of Buffer WASH. DNA was eluted into 50 ml falcon tubes by adding 5 ml/15 ml of Buffer ELU to the columns and allowing these to elute by gravity flow. The collected DNA was precipitated by adding 3.5 ml/10.5 ml of room temperature isopropanol and vortexing thoroughly prior to centrifugation at 15000 g for 30 minutes at 4°C. The supernatant was discarded and the clear DNA pellets were gently re-suspended in 70% ethanol and transferred to sterile eppendorf tubes. These were centrifuged at 14000 rpm for 10 minutes and the supernatant was discarded, following which the DNA pellet was air dried and resuspended in 50 µl/100 µl of Buffer TE.

The DNA concentration was determined using the Nanodrop ND-1000 Spectrophotometer (Labtech International) at OD260 (an OD of 1 at 260 nm corresponds to 50 µg/ml of double-stranded DNA). Plasmid stocks were diluted to a standard 1 µg/µl concentration when possible.

2.6 Cell culture

2.6.1 Cell lines and maintenance

The human cell lines used in this study are listed in table 2.4. The adherent 293T, HeLa and TZM-bl cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen), supplemented with 10% foetal calf serum (FCS) GIBCO), heat-inactivated for 30 minutes at 56°C, and 1% penicillin-streptomycin (Invitrogen). Cells were cultured in 10 cm tissue culture dishes and passaged every 2 days by washing in 1X phosphate buffer saline (PBS) (Invitrogen) and detaching cells from the plate by adding 3 ml of the Trypsin replacement, TrypLE Express (1X), Phenol Red reagent (Invitrogen). Cells were incubated at 37°C for approximately 10 minutes and, depending on the confluency required for experiments, were split into new plates containing fresh DMEM media pre-warmed to 37°C. Suspension HUT78, CEM and Jurkat T cell lines were cultured in Roosevelt Park Memorial Institute medium (RPMI) (Invitrogen), supplemented with 10% FCS and 1% penicillin-streptomycin as described. Cells were cultured in tissue culture flasks and passaged every 2 days by splitting cells into new flasks and adding fresh RPMI media pre-warmed to 37°C.

Table 2.4. Human cell lines.

Cell line	Origin	Type
293T	Embryonic kidney fibroblasts expressing the SV40 large T antigen	Adherent
HeLa/HeLa-HA*	Cervical adenocarcinoma epithelial cells	Adherent
TZM-bl	HeLa cells expressing CD4, CXCR4, CCR5 and the HIV-1 Tat inducible *lacZ* gene	Adherent
HUT78	T cell leukemia cells	Suspension
CEM/CEM-SS**	T cell leukemia cells	Suspension
JURKAT	T cell leukemia cells	Suspension

* HeLa-HA cells are a clonal derivative of HeLa cells that support retrotransposition
** CEM-SS cells are a clonal derivative of CEM cells

2.6.2 Primary cells, maintenance and IFNα treatment

Primary CD4+ T cells were cultured in RPMI supplemented with 10% FCS and 1% penicillin-streptomycin as described, as well as IL-2 (100 U/ml) (BD Pharmingen) and PHA (1 μg/ml) (Oxoid) or soluble anti-CD3 (1 μg/ml) (BD Pharmingen) and anti-CD28 (1 μg/ml) (BD Pharmingen). MDDCs and MDMs were cultured by Dr. Fransje Koning in RPMI supplemented with 10% FCS and 1% penicillin-streptomycin as described. To obtain MDDCs, CD14+ monocytes were treated with GM-CSF (10 ng/ml) (Miltenyi Biotec) and IL-4 (100 ng/ml) (R&D Systems) for 5 days, and for MDMs CD14+ monocytes were allowed to adhere to the plate for 3hrs in RPMI medium supplemented only with 1% penicillin-streptomycin, following which 10% FCS and GM-CSF (100 ng/ml) was added. For IFNα experiments, CD4+ T cells, MDDCs and MDMs pre-treated as described were cultured in the presence of IFNα (1000 U/ml) (PBL InterferonSource) for the indicated length of time.

All cell lines and primary cells were maintained at 37°C with 5% CO_2 and all tissue culture plastics were purchased from Corning Incorporated.

2.6.3 Primary cell isolations

For primary CD4+ T cell isolations, Dr. Anna Le Tortorec isolated PBMCs from the blood of healthy donors using Lymphoprep (Axis-Shield), and also removed the CD14+ monocytes. Remaining cells were provided and the CD4+ T cells were isolated using a CD4+ T cell isolation kit (Miltenyi Biotec), which isolates CD4+ T cells by depleting non-target cells. Briefly, the number of cells was determined using a haemocytometer and these were pelleted by centrifugation at 300 g for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 40 µl of MACS buffer (1X PBS, 0.5% BSA and 2 mM EDTA, pH 7.2) per 10^7 of total cells. 10 µl of the CD4+ T cell biotin-antibody cocktail was added per 10^7 of total cells and incubated at 4°C for 10 minutes. 30 µl of MACS buffer per 10^7 of total cells was added, following which 20 µl of the anti-biotin microbeads was added per 10^7 of total cells and incubated at 4°C for 15 minutes. Cells were washed in 1-2 ml of MACS buffer per 10^7 of total cells and centrifuged as described. The supernatant was discarded and the cell pellet was resuspended in 500 µl of MACS buffer per 10^8 of total cells. CD4+ T cells were isolated by magnetic separation using an LS column. For this, the LS column was rinsed with 3 ml of MACS buffer and allowed to empty by gravity flow. The cell suspension was applied to the column, and the flow-through containing unlabelled enriched CD4+ T cells was collected. The column was again washed 3X with 3 ml of MACS buffer, and the flow-through was collected. The CD4+ T cells were counted and 10 x 10^6 cells were transferred to each flask for experiments. 2 x 10^6 cells were harvested for analysis on indicated days. CD14+ monocytes were isolated by Dr. Fransje Koning by positive selection using CD14 MicroBeads (Miltenyi Biotec) and the experiments were also performed by Dr. Fransje Koning. Cell lysates were provided for analysis as described.

2.6.4 Freezing and thawing cell lines

Semi-confluent plates or flasks of cells were pelleted at 1200 rpm for 5 minutes. Cell pellets were resuspended in freezing medium (10% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) diluted in FCS) and aliquoted into cryovials on ice. Cells were immediately stored at -80°C or transferred to liquid nitrogen the next day for long-term storage. Cells were thawed by incubating frozen vials at 37°C for approximately 1 minute and immediately transferring cells to 5 ml of pre-warmed medium. Cells were washed by centrifuging at 1200 rpm for 5 minutes, resuspended in fresh media and

transferred to 10 cm dishes (adherent cells) or tissue culture flasks (suspension T cells) for culture.

2.7 Transfections

2.7.1 Plasmid transfections

HeLa or 293T cells (2 x 10^5 or 1 x 10^5 cells (latter for immunofluorescence)) were plated 24hrs prior to plasmid transfection. Alternatively, 70% confluent 10 cm tissue culture plates of 293T cells were transfected for some experiments. For transfection of 293T cells, a polyethyleneimine (PEI, Polysciences) mastermix was prepared (16 µl PE1 (1 mg/ml) and 180 µl of serum-free DMEM for transfection of a single well in a 6-well dish, or 50 µl of PEI and 550 µl of serum-free DMEM for transfection of a 10 cm tissue culture dish) and incubated for 5 minutes at room temperature. DNA amounts as stated in the figure legends were incubated with 196 µl or 600 µl, respectively, of the PEI mastermix for 20 minutes at room temperature and the transfection mixture was then added to 293T cells drop-by-drop. For transfection of HeLa cells, a Fugene 6 (Roche) mastermix was prepared (3 µl FuGENE to 1 µg DNA ratio diluted in Optimem (Invitrogen) to a total volume of 100 µl for transfection of a single well in a 6-well dish) and incubated for 5 minutes at room temperature. DNA amounts as stated in the figure legends were incubated with 100 µl of the Fugene 6 mastermix and the transfection mixture was then added to HeLa cells drop-by-drop as described. Fresh media was replaced approximately 6hrs post-transfection.

2.7.2 siRNA transfections

For siRNA transfections (50 µM or 75 µM diluted in RNase free ddH_2O (Ambion Life Technologies)), HeLa cells were seeded (1 x 10^5) 2hrs before and allowed to adhere to the plate. A Dharmafect1 (Dharmacon) master mix was prepared (1 µl Dharmafect1 and 49 µl Optimem for transfection of a single well in a 24-well dish) and incubated for 5 minutes at room temperature. Separately, an siRNA mixture was prepared (0.66 µl siRNA and 49 µl Optimem) and 50 µl of the Dharmafect1 mastermix was added to each siRNA mixture, following which these were incubated for 20 minutes at room temperature. Cell culture media was replaced with 400 µl of fresh media and 100 µl of the transfection mixture. On Day 2 transfected cells were replaced with fresh media, and on Day 3 cells were split 1:4 into new 24-well plates, allowed to adhere to the plate and transfected again with siRNAs as described for the first day. Approximately 12-

15hrs post-transfection cells were transfected with plasmid DNA using the Fugene 6 transfection reagent as described (section 2.7.1), and approximately 24hrs later cells were harvested for analysis as required.

2.8 Transductions for generation of stable cell lines

Lentiviral vectors for the production of HeLa, 293T and HUT78 non-silencing control and MOV10 KD stable cell lines were produced by transfecting 293T cells in 10 cm tissue culture dishes as described (section 2.7.1) with p8.91 (8 μg), the GIPZ lentiviral vector expressing either the non-silencing control shRNA or MOV10-specific shRNA (8 μg) and pVSV-G (2 μg). Fresh media was replaced the following day, and approximately 48hrs post-transfection lentiviral vectors were harvested and filtered through a 0.45 μM filter (Millipore). Cells to be transduced were plated 24hrs prior to transduction (2 x 10^5 cells). For transduction of the cells, 1 ml of the cell culture medium was replaced with 1 ml of vector-containing supernatant. Fresh media was replaced approximately 24hrs post-transduction. The following day, cell supernatant was replaced with media containing puromycin dihydrochloride (1 μg/ml) (Sigma-Aldrich). Cells were maintained under selection and analysed when untransduced control cells died (approximately 3 days from selection). The GIPZ lentiviral vectors also express GFP allowing the transduction efficiency to be determined by checking the percentage of GFP+ cells.

2.9 Infectivity assays

2.9.1 HIV-1 (subgenomic and provirus)

HeLa and 293T parental cells or non-silencing control and MOV10 KD cells (2 x 10^5 cells) were co-transfected with plasmids as stated in the figure legends using the protocol described (section 2.7.1). Approximately 48hrs post-transfection, virus-containing supernatant was harvested and filtered through a 0.45 μM filter, and cells were washed in 1X PBS and lysed in 500 μl of radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) for analysis by immunoblotting. 800 μl of the filtered viral supernatant was purified through 200 μl of a 20% sucrose cushion (diluted in 1X PBS) by centrifugation at 14000 rpm for 2hrs at 4°C and prepared for analysis by

immunoblotting where described. 100 µl of the filtered viral supernatant was diluted in 400 µl of 0.5% Triton X-100 for analysis by $p24^{Gag}$ ELISA (Perkin-Elmer)

2.9.1.1 $p24^{Gag}$ ELISA

For 293T experiments, the 1:5 diluted viral supernatant was diluted further to 1:250 and for HeLa experiments these were diluted further to 1:10. A 96-well plate pre-coated with a monoclonal antibody against HIV-1 $p24^{Gag}$ (in 0.01% Proclin-300 preservative) was washed 6X in ELISA wash buffer (0.05% Tween-20 in PBS with 0.1% 2-chloroacetamide as a preservative) using a plate washer (BioTek Elx50). 200 µl of each $p24^{Gag}$ standard (10000, 2000, 500, 150, 60 and 25 pg/ml), prepared using a $p24^{Gag}$ positive control (200 ng/ml $p24^{Gag}$ in PBS, plus BSA, Triton-X 100 and 0.1% sodium azide as a preservative) diluted in the lysis buffer (0.5% Triton X-100 in PBS plus inert blue dye and 0.002% sodium azide as a preservative), was added to the plate. A lysis buffer only negative control and an empty well was also included (A1). 200 µl of the samples were then added to wells and the plate was incubated at 37°C for 1hr. The plate was washed 6X in ELISA wash buffer prior to adding 100 µl of the detector antibody (biotinylated rabbit polyclonal anti-$p24^{Gag}$ antibody in PBS containing animal sera, casein and human serum non-reactive for Hepatitis B surface antigen and antibodies to HIV-1, HIV-2, and HCV, plus 0.2% Proclin-300 and 0.1% sodium azide as a preservative) to all wells except the empty A1 well. The plate was incubated at 37°C for 30 minutes, following which the plate was washed 6X in ELISA wash buffer. The streptavidin-HRP concentrate (in citrate buffer with BSA, detergent and 0.5% 2-chloroacetamide as a preservative) was diluted 1:100 into the streptavidin-HRP diluent (PBS with BSA, 0.05% Tween-20 and 0.5% 2-chloroacetamide as a preservative), and 100 µl of this mixture was added to each well except A1. The plate was incubated at 37°C for 30 minutes and then washed 6X in ELISA wash buffer. An ortho phenylenediamine-HCL (OPD) tablet was dissolved in 11 ml of substrate diluent (citrate buffer containing 0.03% hydrogen peroxide and 0.002% sodium stannate as a stabiliser) and 100 µl of this solution was added to each well including A1. The plate was incubated at room temperature for 10-15 minutes in the dark, following which the reaction was stopped by adding 100 µl of the STOP solution (4N sulfuric acid). The plate was read using a Benchmark Plus microplate spectrophotometer (Bio-Rad) at dual (subtractive) wavelengths of 405 nm and 630 nm. Values were analysed using the Microplate Manager 5.2.1 software (Bio-

Rad) to determine the $p24^{Gag}$ concentration (pg/ml). All the reagents described were provided in the $p24^{Gag}$ ELISA kit.

2.9.1.2 TZM-bl assay

TZM-bl cells (1×10^5) were plated 24hrs prior to infection. These cells express the lacZ gene from under the HIV-1 Tat protein inducible HIV-1 LTR. Equal amounts of virus normalised by the $p24^{Gag}$ concentration (1-5 ng) was used to infect the TZM-bl cells. Approximately, 30hrs post-infection cells were lysed in 100 µl Tropix Lysis solution (100 mM potassium phosphate pH 7.8, 0.2% Triton X-100) (Applied Biosystems) for approximately 5 minutes at room temperature, and the plates were then frozen overnight at -80°C. Plates were thawed and lysed cells were harvested. 20 µl of the lysates were added to a 96-well white polystyrene plate. The β-galactosidase substrate was prepared by diluting the Tropix Galacton-Star substrate 1:50 in the reaction buffer diluent (100 mM sodium phosphate pH 7.5, 1 mM MgCl2, 5% Sapphire-II enhancer) (Applied Biosystems) and adding 100 µl of this solution to each well containing the cell lysates. β-galactosidase activity was measured using a luminescence counter (Perkin-Elmer) and Wallac 1420 Workstation software.

2.9.2 HIV-1 infection of producer cells

VSV-G pseudotyped HIV-1_{NL4-3} strain virus was produced by transfecting a 10 cm tissue culture plate of 293T cells with plasmids as stated in the figure legends using the protocol described (section 2.7.1). The virus concentration was determined by $p24^{Gag}$ ELISA (section 2.9.1.1). The MOI of the virus stock was determined with a $p24^{Gag}$ intracellular FACS stain (Dako). Briefly HeLa or 293T cells were infected with a range of viral concentrations in a total volume of 1 ml for approximately 4hrs, following which the cells were washed 3X in 1X PBS and replaced with 2 mls of fresh media. Approximately, 48hrs post-infection the supernatant was removed and 1 ml of the TrypLE Express reagent was added to detach the cells from the plate. Cells were transferred to an eppendorf tube and washed in FACS buffer (1X PBS plus 5% FCS by centrifuging at 8000 rpm for 5 minutes. The supernatant was discarded and the cell pellets were resuspended in 100 µl of Buffer A and incubated at room temperature for 10-15 minutes. This step inactivates the virus. Cells were washed in 1.5 ml FACS buffer by centrifuging as described. The supernatant was discarded and the cells pellets

were resuspended in 100 µl of Buffer B and 2 µl of the KC57-RD1 $p24^{Gag}$ monoclonal antibody (Beckman Coulter). These were incubated for 20 minutes at room temperature in the dark and then washed 4X in FACS buffer by centrifuging as described. The cell pellets were resuspended in 200 µl of FACS buffer and analysed using a FACS Canto II Flow Cytometry System (BD Biosciences). Mock-infected cells were also stained and included in the analysis as a negative control. HeLa or 293T non-silencing control and MOV10 KD cells (2×10^5) were infected with 25 ng (MOI 0.3) of the VSV-G pseudotyped HIV-1_{NL4-3} strain virus in a total volume of 1 ml for approximately 4hrs as described for the $p24^{Gag}$ intracellular FACS stain. Approximately 48hrs post-infection, the virus-containing supernatant was harvested and filtered, and the cells were lysed as described (section 2.9.1). The virus concentration was determined by $p24^{Gag}$ ELISA (section 2.9.1.1) and the virion infectivity was measured by TZM-bl assay (section 2.9.1.2).

2.9.3 HIV-1 spreading replication

HUT78 non-silencing control and MOV10 KD cells were generated as described (section 2.8). Cells (1×10^6 cells) were pelleted by centrifuging at 300 g for 5 minutes and resuspended in 1 ml of medium containing 100 ng of wild-type HIV-1_{NL4-3} strain virus produced in 293T cells. These were incubated at 37°C for 2hrs, following which the cells were washed in 5 ml of 1X PBS by centrifuging as described. The cells pellets were resuspended in 10 ml of medium and transferred to flasks for culturing. Cells were passaged every 2 days by replacing 5 ml of the culture with fresh media, and the 5 ml cell suspension removed was pelleted by centrifugation as described. 100 µl of the virus-containing supernatant was diluted in 400 µl of 0.5% Triton X-100 for analysis by $p24^{Gag}$ ELISA (section 2.9.1.1). The cells were resuspended in RIPA buffer for analysis by immunoblotting.

2.9.4 SIVmac and HIV-1 vectors

293T parental or non-silencing control and MOV10 KD cells (2×10^5 cells) were transfected with plasmids as stated in the figure legends using the protocol described (section 2.7.1). Approximately 48hrs post-transfection, vector-containing supernatant was harvested and filtered through a 0.45 µM filter. 293T cells (1×10^5 cells) were plated 24hrs prior to transduction with 500 µl of the vector-containing supernatant.

Approximately 24hrs post-transduction, the cell supernatant was removed and cells were washed in 1X PBS, following which the TrypLE Express reagent was added to detach cells from the plate. Cells were fixed in 4% PFA (Electron Microscopy Sciences) (diluted in 1X PBS) for 10 minutes at room-temperature. Cells were washed 3X in 1X PBS by centrifuging at 1200 rpm for 5 minutes. Cell pellets were resuspended in 200 µl of 1X PBS and analysed using a FACS Canto II Flow Cytometry System (BD Biosciences) to determine the percentage of GFP+ cells.

2.9.5 SIVmac and HIV-2 provirus

293T cells (2 x 10^5) were transfected with plasmids as stated in the figure legends using the protocol described (section 2.7.1). Approximately 48hrs post-transfection, virus-containing supernatant was harvested and filtered through a 0.45 µM filter. 800 µl of the filtered viral supernatant was purified through a 20% sucrose cushion by centrifugation at 14000 rpm for 2hrs at 4°C and prepared for analysis by immunoblotting. Cells were lysed in RIPA buffer and analysed by immunoblotting. For HIV-2, TZM-bl cells (1 x 10^5 cells) were plated and infected with 500 µl of the virus-containing supernatant 24hrs later. Approximately 24hrs post-infection, cells were lysed and analysed by TZM-bl assay (section 2.9.1.2) to quantify the production of infectious virus.

2.9.6 MLV and M-PMV

293T parental or non-silencing control and MOV10 KD cells were transfected with plasmids as stated in the figure legends using the protocol described (section 2.7.1). Approximately 48hrs post-transfection, virus-containing supernatant was harvested and filtered through a 0.45 µM filter. For M-PMV, 800 µl of the filtered viral supernatant was purified through a 20% sucrose cushion by centrifugation at 14000 rpm for 2hrs at 4°C and prepared for analysis by immunoblotting. Cells were also lysed in RIPA buffer and analysed by immunoblotting. TZM-bl cells (1 x 10^5 cells) were plated and infected with 500 µl of the MLV or M-PMV-containing supernatant 24hrs later. Approximately 24hrs post-infection, cells were lysed and analysed by TZM-bl assay (section 2.9.1.2) to quantify the production of infectious MLV and M-PMV virions.

2.10 Quantification of HIV-1 viral RNA packaging

HeLa or 293T cells were transfected in 10 cm tissue culture dishes with plasmids as stated in the figure legends using the protocol described (section 2.7.1). Approximately 48hrs post-transfection, virions were harvested and filtered through a 0.45 μM filter. 100 μl of the virus-containing supernatant was diluted in 400 μl of 0.5% Triton X-100 for analysis by $p24^{Gag}$ ELISA (section 2.9.1.1). The remaining filtered viral supernatant was treated with RQ1 DNase (20 U/ml) (Promega) and magnesium chloride (10 mM final concentration) for 2-3hrs at 37°C. Equal amounts of virus normalised by the $p24^{Gag}$ concentration was purified through a 20% sucrose cushion by ultracentrifugation at 28000 rpm for 1hr and 15 minutes at 4°C. RNA was extracted from the viral pellet using the QIAgen RNeasy Mini kit (QIAgen). Briefly, the supernatant was carefully removed and the viral pellet was lysed in 350 μl of Buffer RLT (contains guanidine thiocyanate). The lysate was homogenised by transferring it to a QIA shredder spin column and centrifuging at 14000 rpm for 2 minutes. The flow-through was collected in a 2 ml collection tube. 350 μl of 70% ethanol was added to the homogenised lysate and mixed thoroughly by pipetting. Approximtely 700 μl of the sample was transferred to an RNeasy spin column and centrifuged at 10000 rpm for 15 seconds. The flow-through was discarded. For on-column DNase digestion, 350 μl of Buffer RW1 (contains guanidine thiocyanate and ethanol) was added to wash the column by centrifugation at 10000 rpm for 15 seconds and the flow-through was discarded. 100 μl of RQ1 DNase was added to the column and incubated at room temperature for 30 minutes. The column was washed again with 350 μl of Buffer RW1 and centrifugation as described. The flow-through was discarded. Following this the column was washed with 500 μl of Buffer RPE (contains ethanol) and centrifuged as described. The flow-though was discarded and the same step was repeated, however, this time with centrifugation at 10000 rpm for 2 minutes to remove any traces of ethanol. RNA was eluted by adding 30 μl of RNase-free water to the column and centrifuging at 10000 rpm for 1 minute. The RNA concentration was determined using the Nanodrop ND-1000 Spectrophotometer at OD260 (an OD of 1 at 260 nm corresponds to 40 μg/ml of RNA).

For the reverse-transcription (RT)-PCR reaction, equal amounts of RNA (within the range 0.002-0.2 μg/ μl) diluted in RNase-free water to a total volume of 25 μl was

added to 25 µl of the 2X RT mastermix, prepared using the High Capacity cDNA reverse transcription kit (Applied Biosystems). The RT mastermix was made up of 5 µl of 10X random primers, 5 µl of 10X RT buffer, 2 µl of 25X dNTP (100mM), 2.5 µl of the MultiScribe RT enzyme (50 U/ µl) and 10.5 µl of RNase-free water. Duplicate samples minus the RT enzyme were included to ensure efficient removal of DNA contamination by RQ1 DNase digestion. The RT reaction was performed using the 2720 Thermal Cycler (Applied Biosystems) according to conditions outlined in table 2.5. The level of HIV-1 viral RNA packaged into virions was determined by qPCR (section 2.12) using a primer-probe set that recognises sequences within the Gag p6 region (oNS172f, oNS173r and oNS174p FAMTAMRA). The primer-probe sequences are listed in table 2.7.

Table 2.5. RT-PCR reaction conditions.

	Temperature	Time
1	25° C	10 minutes
2	37° C	120 minutes
3	85° C	5 minutes
4	4° C	Hold

2.11 Quantification of HIV-1 reverse transcripts

293T cells were transfected in 10 cm tissue culture dishes with plasmids as stated in the figure legends using the protocol described (section 2.7.1). Approximately 48hrs post-transfection, virions were harvested and filtered through a 0.45 µM filter. 100 µl of the virus-containing supernatant was diluted in 400 µl of 0.5% Triton X-100 for analysis by $p24^{Gag}$ ELISA (section 2.9.1.1). HUT78 cells (1 x 10^6 cells) were spin-infected with 20 ng of wild-type HIV-1_{NL4-3} strain virus at 2000 g for 2hrs at 4°C. The supernatant was aspirated and cells were washed 3X in cold 1X PBS by centrifuging at 2000 g for 10 minutes. The cells pellets were resuspended in media and cultured. Cells were harvested at the indicated time-points and pelleted by centrifugation at 2000 g for 10 minutes. DNA was extracted from the cell pellet using the QIAgen DNeasy kit (QIAgen). Briefly, the pelleted cells were lysed in 200 µl 1X PBS, 20 µl Proteinase K and 200 µl of Buffer AL (contains guanidine hydrochloride) at 70°C for 10 minutes.

200 µl of 96-100% ethanol was added to the samples and mixed thoroughly by vortexing. The mixture was added to a DNeasy Mini spin column and centrifuged at 8000 rpm for 1 minute. The flow-through was discarded. 500 µl of Buffer AW1 (contains guanidine hydrochloride and ethanol) was added to the column and centrifuged as described. The flow-through was discarded. 500 µl of Buffer AW2 (contains ethanol) was then added to the column and centrifuged at 14000 rpm for 3 minutes. DNA was eluted by adding 200 µl of Buffer AE to the column and spinning at 8000 rpm for 1 minute. The DNA concentration was determined using the Nanodrop ND-1000 Spectrophotometer at OD260. To digest input plasmid DNA, equal amounts of extracted DNA (diluted in nuclease-free water to a total volume of 20.5 µl) was incubated with 2 µl of DpnI enzyme and 2.5 µl of the appropriate buffer at 37°C for 2hrs. Minus strand strong stop DNA levels were quantified by qPCR (section 2.12) using a primer-probe set that recognises sequences between R and the U5-PBS junction (oHC64, oHC65 and oHC66 FAMTAMRA). The primer-probe sequences are listed in table 2.7.

2.12 Quantitative PCR (qPCR)

The qPCR reactions were performed in triplicate. The mastermix was prepared with 5 µl TaqMan Universal PCR mastermix (Applied Biosystems), 1 µl each of the forward and reverse primer (9 pmol/µl) and 1 µl of the probe (2.5 pmol/µl). 8 µl of the qPCR mastermix was added to 2 µl of either diluted or neat sample cDNA or a serial dilution of the pHIV-1_{NL4-3} plasmid standard (nuclease-free water only, 2, 5, 10, 50, 100, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 molecules per 2 µl diluted in salmon sperm DNA (20 ng/ml)) in a 384-well clear optical reaction plate (Applied Biosystems). For TaqMan gene expression assays, the mastermix was prepared with 10 µl of the TaqMan Universal PCR mastermix, 1 µl of the TaqMan assay, 4 µl of the cDNA and 5 µl of nuclease-free water. For relative quantification, samples were normalised to GAPDH mRNA levels. The qPCR reaction was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) according to PCR conditions outlined in table 2.6, and analysed using the SDS2.3 software (Applied Biosystems). The primer-probe set sequences used for qPCR in this study are listed in table 2.7.

Table 2.6. qPCR reaction conditions.

	Temperature	Time
1	50° C	2 minutes
2	95° C	10 minutes
3	95° C	15 seconds
4	60° C	1 minute
5	Go to step 2, repeat 40 times	

Table 2.7. qPCR primer-probe set sequences.

Primer name	Sequence
oNS172f	GGCCAGGGAATTTTCTTCAGA
oNS173r	TTGTCTCTTCCCCAAACCTGA
oNS174p FAMTAMRA	ACCAGAGCCAACAGCCCCACCAGA
oHC64	TAACTAGGGAACCCACTGC
oHC65	GCTAGAGATTTTCCACACTG
oHC66 FAMTAMRA	ACACAACAGACGGGCACACACTA

2.13 Immunoblotting

2.13.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Cells lysed in RIPA buffer were diluted 1:2 in 2X dissociation buffer (125mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.2% Bromophenol blue and ddH_20. 10% β-mercaptoethanol was added just prior to use). Denaturing gels were cast using a large vertical electrophoresis system (CBS Scientific) (Either 7% (proteins > 80 kda) or 10% (proteins < 80 kda) acrylamide bis/solution 37.5:1 (Bio-Rad), 375 mM Tris-HCL pH 8.8, 0.1% SDS, 0.1% ammonium persulphate, 0.0004% N, N, N', N'-tetramethylethylenediamine (TEMED) in ddH_20). The gels were leveled and prevented from drying by layering 0.1% SDS on top of the running gel and this was removed prior

to adding the stacking gel (4.5% acrylamide bis/solution, 125 mM Tris-HCL pH 6.8, 0.1% SDS, 0.1% ammonium persulphate, 0.001% TEMED and ddH_20). Samples were boiled for 10 minutes prior to loading. A protein ladder was also loaded as a marker for protein size (Benchmark Prestained protein ladder) (NEB).

2.13.2 Immunoblot analysis

Proteins were transferred onto 0.45 μm nitrocellulose membranes (Anachem) at 60 volts for approximately 4hrs in a large electrophoretic blotting system (CBS Scientific) containing transfer buffer (27.6 mM Tris Base, 0.2 M glycine, 20% methanol diluted in ddH_20, pH > 8.8). Membranes were blocked in 1% milk solution (1% milk powder in 1X PBS and 0.1% Tween-20 (Fisher Scientific)) at room temperature for 1hr, following which membranes were incubated with the primary antibody in milk solution for approximately 1-2hrs at room temperature. The membranes were washed 3X in wash buffer (1X PBS and 0.1% Tween-20) and incubated with 800λ Infrared IRDye conjugated secondary antibodies in milk solution for 1hr at room temperature in the dark. Subsequently, the membranes were washed again as described and imaged using a Li-cor Odyssey infrared scanner (Li-cor Biosciences). Primary and secondary antibodies used are listed in table 2.8 and 2.9 (IB).

2.14 Immunofluorescence

HeLa cells (1×10^5) cells were plated onto coverslips and transfected with plasmids as stated in the figure legends using the protocol described (section 2.7.1). Where described, HeLa non-silencing control and MOV10 KD cells (1×10^5) were also plated, however, these cells were not transfected with any plasmids. Approximately 24hrs later cells were washed 3X in 1X PBS and fixed with 4% PFA for 10 minutes at room temperature. Cells were washed again as described and permeabilised with 0.2% Triton X-100 for 15 minutes at room temperature. Subsequently, cells were blocked and quenched in NGB buffer (50 mM NH_4Cl, 2% goat serum, 2% BSA and 0.05% sodium azide in PBS) for 1hr at room temperature, following which the primary antibody was diluted in NGB buffer and added to the cells for 1-2hrs at room temperature. Coverslips were washed as described before incubation with the appropriate Alexa Fluor conjugated secondary antibody diluted in the NGB buffer for 1hr at room temperature in the dark. The secondary antibody was removed and cells were incubated for an additional 1 minute with the DAPI stain (30 nm final concentration) (Molecular

Probes). Coverslips were washed as described and mounted onto glass slides (Fisher Scientific) using 10 µl of Mowiol mounting media (Calbiochem). The slides were dried overnight in the dark and images were collected using a TCS SP2 AOBS confocal laser-scanning microscope (DM IRE2: Leica), with a 63X oil objective lens and processed with the LSC software (version 2.02, Leica) and Adobe Photoshop (CS5.1). Primary and secondary antibodies used are listed in table 2.8 and 2.9 (IF).

Table 2.8. Primary antibodies for immunoblotting and immunofluorescence.

Antibody	Species	Dilution	Source
Anti-MOV10	Rabbit	1/1000 (IB, IF)	Proteintech
Anti-A3G/A3A	Rabbit	1/1000 (IB)	(Newman et al., 2005)
Anti-TRIM22	Rabbit	1/250 (IB)	Sigma-Aldrich
Anti-Hsp90	Rabbit	1/3000 (IB)	Santa Cruz
Anti-DDX6	Rabbit	1/500 (IF)	Cambridge Biosciences
Anti-Ge1	Mouse	1/500 (IF)	Santa Cruz
Anti-T7	Mouse	1/10000 (IB, IF)	Novagen
Anti-myc (9E10)	Mouse	1/1000 (IB, IF)	(Evan et al., 1985)
Anti-HIV-1 $p24^{Gag}$ (24.2)	Mouse	1/1000 (IB)	(Fouchier et al., 1997)
Anti-HIV-1 $p24^{Gag}$ (183) (HIV-2, SIVmac)	Mouse	1/100 (IB)	Kind gift from Dr. Stuart Neil
Anti-HIV-1 $p24^{Gag}$ (UP598)	Rabbit	1/500 (IF)	(Simon et al., 1997)
Anti-HIV-1 $p17^{Gag}$ (UP595)	Rabbit	1/500 (IF)	(Swanson et al., 2004)
Anti-M-PMV $p27^{Gag}$ (78 S-136)	Goat	1/200 (IB)	Microbiological Association

* IB = immunoblotting

* IF = immunofluorescence

Table 2.9. Secondary antibodies for immunoblotting and immunofluorescence.

Antibody	Conjugation	Dilution	Source
800nmλ Goat α-mouse	IRDye	1/10000 (IB)	Li-cor Biosciences
800nmλ Goat α-rabbit	IRDye	1/10000 (IB)	Li-cor Biosciences
800nmλ Donkey α-goat	IRDye	1/10000 (IB)	Li-cor Biosciences
488nmλ Goat α-mouse	Alexa Fluor 488	1/500 (IF)	Molecular Probes
594nmλ Goat α-rabbit	Alexa Fluor 594	1/500 (IF)	Molecular Probes

* IB = immunoblotting

* IF = immunofluorescence

2.15 Retrotransposition assay

Parental HeLa-HA or non-silencing control and MOV10 KD HeLa-HA cells (2 x 10^5 cells) were transfected with plasmids as stated in the figure legends using the protocol described (section 2.7.1). The next day 1 ml of the TrypLE Express reagent was added to detach the cells from the plate, and cells were transferred into 10 cm tissue culture dishes. The following day the cell culture was selected with G418 (1 mg/ml final concentration) (Sigma-Aldrich). Approximately 5 days later fresh media containing G418 was replaced. Approximately 12-15 days later, when the control untransfected cells had died, cells were washed 3X in 1X PBS and fixed in 4% PFA for 10 minutes at room temperature. Cells were washed again as described, following which the cells were stained in 0.4% Giemsa stain (dissolved in 70% ethanol) (Sigma-Aldrich) for 40 minutes at room temperature. The cells were washed 3X in ddH_20 and G418-resistant cell colonies were counted.

2.16 Luciferase assays

Parental HeLa-HA or non-silencing control and MOV10 KD HeLa-HA cells (1 x 10^5 cells) were transfected with plasmids as stated in the figure legends using the protocol described (section 2.7.1). Luc assays were performed using the Dual Luciferase Reporter kit (Promega). For this, approximately 24hrs post-transfection, cells were washed in 1X PBS before being lysed in 100 µl of 1X Passive Lysis Buffer (diluted in

ddH_20) at room temperature for 15 minutes with gentle shaking. The Luciferase Assay Reagent II (LAR II) was prepared by dissolving the Luciferase Assay substrate in the Luciferase Assay Buffer II provided. 100 µl of LAR II was added to 20 µl of the cell lysate in a white polystyrene 96-well plate and mixed well. Firefly activity was measured immediately using a luminescence counter and the Wallac 1420 Workstation software. Following these readings, 100 µl of the Stop & Glo reagent, prepared by diluting the Stop & Glo substrate 1:50 into the Stop & Glo Buffer provided, was added to the wells to quench firefly Luc activity and measure Renilla Luc activity. Readings were taken as described.

CHAPTER 3

RESULTS

MOV10 overexpression restricts the replication of retroviruses and retrotransposons

3.1 Introduction

Human MOV10 regulates the replication of several RNA viruses (Haussecker et al., 2008; Li et al., 2011; Schoggins et al., 2011). This function appears to be evolutionarily conserved as MOV10 homologs are necessary for the inhibition of viruses and endogenous retroelements (Dalmay et al., 2001; Frost et al., 2010; Olivieri et al., 2010; Zheng et al., 2010) (chapter 1 section 1.13.2). Furthermore, MOV10 interacts with members of the APOBEC3 family that inhibit the replication of both retroviruses and retrotransposons (Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008) (chapter 1 sections 1.8.2.8 to 1.8.2.10), and also associates with miRNA and siRNA-mediated post-transcriptional RNA silencing pathways, which too have been implicated in the regulation of retroelements (Meister et al., 2005) (chapter 1 section 1.12.5). MOV10 and members of the APOBEC3 and AGO family co-localise in P bodies and redistribute to SGs under conditions of stress (Meister et al., 2005; Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). Reports have identified P bodies and SGs, as well as individual components of these RNP microdomains, as playing both restrictive and stimulatory roles in the life cycle of exogenous and endogenous retroelements (Chable-Bessia et al., 2009; Henao-Mejia et al., 2009; Doucet et al., 2010) (chapter 1 sections 1.10.1 and 1.11.1). Considering these associations, the potential capacity of MOV10 to regulate retroelements was established by undertaking side-by-side comparisons of the effect of MOV10 ectopic overexpression on the replication of a panel of retroviruses and retrotransposons.

3.2 HIV-1 subgenomic and full-length provirus assays

MOV10 was identified as an A3F and A3G interacting RBP (Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008), both of which are intrinsic HIV-1 restriction factors (Bishop et al., 2004; Liddament et al., 2004; Wiegand et al., 2004) (chapter 1 section 1.8.2.8). Therefore, the effect of MOV10 overexpression on the production and infectivity of HIV-1 particles was determined initially using a subgenomic system as well as full-length HIV-1. The subgenomic construct (GPV-RRE) was generated from the HIV-1_{NL4-3} provirus and encodes *gag*, *pro*, *pol*, *vif* and part of *vpr*, as well as the RRE directing nuclear export via the CRM1/Rev pathway (Figure 3.1A). GPV-RRE contains only part of the 5' U3 (sequence 3' to TATA box) and complete R and U5 sequences fused to the herpesvirus cytomegalovirus (CMV) promoter, and has a polyadenylation signal derived from the bovine growth hormone (BGH). Additionally, the GPV-RRE construct maintains the 5' splice donor and two 3' splice acceptor sites.

For the subgenomic and provirus assays, HeLa or 293T cells were transfected with either GPV-RRE and a plasmid expressing Rev (pRev), or a plasmid expressing the full-length HIV-1_{NL4-3} provirus (pHIV-1_{NL4-3}) (Adachi et al., 1986) (Figure 3.1B). For MOV10 overexpression experiments, cells were co-transfected with GPV-RRE or pHIV-1_{NL4-3} together with a plasmid expressing MOV10 (pMOV10) or either a GFP or firefly luciferase (Luc) control plasmid (pGFP/pLuc). VLP or virion production was quantified by $p24^{Gag}$ enzyme-linked immunosorbent assay (ELISA) ($p24^{Gag}$ corresponds to CA). For full-length proviral experiments, equal amounts of virus normalised by the $p24^{Gag}$ concentration was added to a TZM-bl reporter cell line expressing a HIV-1 Tat inducible β-galactosidase (β-gal) reporter gene, to establish the effect of MOV10 overexpression on the infectivity of virions produced.

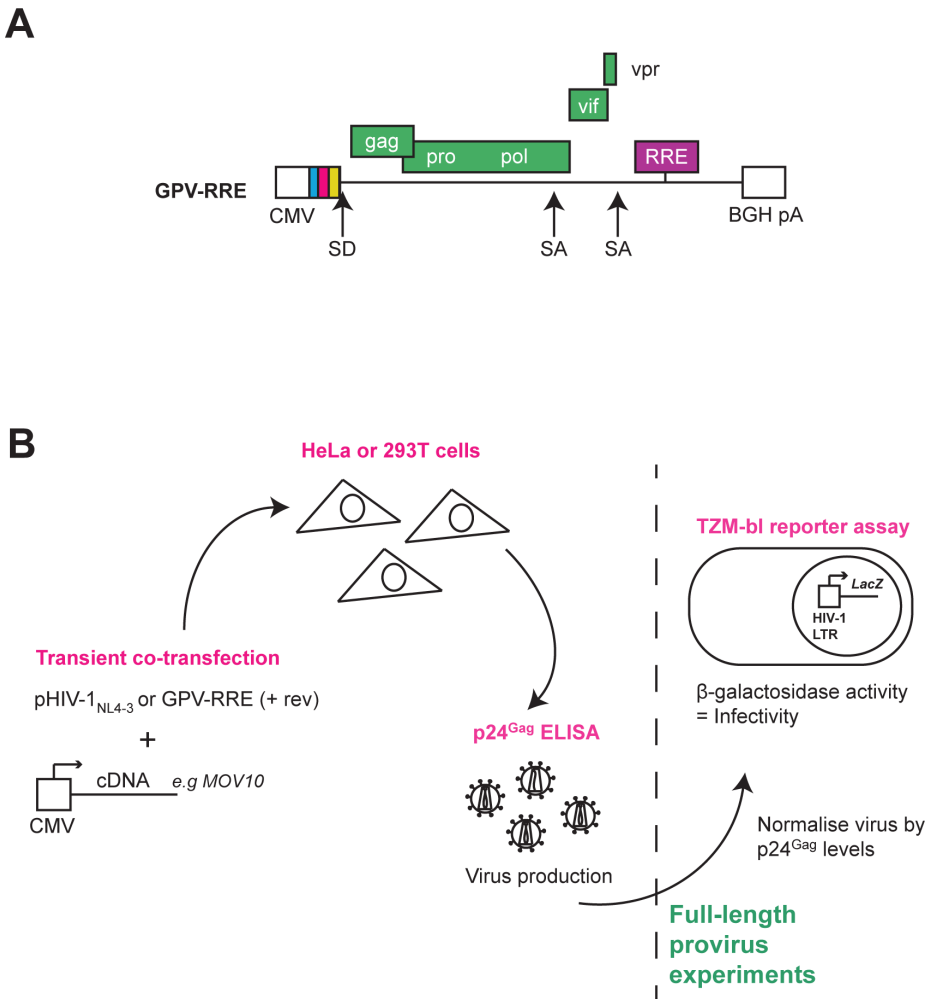


Figure 3.1. HIV-1 subgenomic and full-length provirus assays.

(A) Subgenomic GPV-RRE construct. The GPV-RRE construct was derived from the HIV-1_{NL4-3} provirus and encodes *gag*, *pro*, *pol*, *vif* and part of *vpr*. The RRE, 5' splice donor site and two 3' splice acceptor sites are also retained. Transcription is driven from a cytomegalovirus (CMV) promoter that is fused to part of the 5' U3, and complete R and U5 sequences and the 3' UTR is replaced with a polyadenylation signal from the bovine growth hormone (BGH). **(B) GPV-RRE and proviral assays.** HeLa or 293T cells were co-transfected with either pHIV-1_{NL4-3} (proviral assays) or GPV-RRE and a plasmid expressing the HIV-1 Rev protein (pRev) together with equal concentrations of a plasmid expressing the cDNA of a protein of interest, for example, MOV10. Approximately 48hrs post-transfection virions or virus-like particles (VLPs) in the supernatant were harvested and quantified by $p24^{Gag}$ ELISA. For proviral assays, equal amounts of virus normalised by the $p24^{Gag}$ concentration was added to a TZM-bl reporter cell line expressing a HIV-1 Tat protein inducible β-galactosidase (β-gal) gene. Infectivity was determined by measuring β-gal activity.

3.3 Effect of MOV10 overexpression on HIV-1 virus production and infectivity

3.3.1 Expression of N-terminally and C-terminally-tagged MOV10 proteins

The MOV10 cDNA was cloned into three plasmids with either an N-terminal or C-terminal tag to test the effect of these differential tags on MOV10 protein expression. The plasmids pcDNA3.1-myc-MOV10 (myc-MOV10), pCMV4-MOV10-HA (MOV10-HA) and pT7-MOV10 (T7-MOV10) were transfected into 293T cells, following which cells were harvested and MOV10 expression was analysed by immunoblotting with an anti-MOV10 antibody. MOV10 was expressed efficiently from all three plasmids although MOV10 protein abundance varied with MOV10-HA being expressed at the highest levels (Figure 3.2A).

3.3.2 Overexpression of MOV10 inhibits HIV-1 VLP production

To determine the effect of MOV10 overexpression on VLP production, either myc-MOV10, MOV10-HA, T7-MOV10 or the pGFP control in the relevant plasmid backbone was transfected into 293T cells together with GPV-RRE and pRev. VLP production was quantified by $p24^{Gag}$ ELISA and results showed a potent decrease in VLP production, whereby this was reduced by over 90% with myc-MOV10 and MOV10-HA overexpression, and completely in the case of T7-MOV10 overexpression compared to the pGFP control (Figure 3.2B). The capacity of MOV10-HA to inhibit VLP production was reduced relative to T7-MOV10 despite higher levels of expression (Figure 3.2A), and this may be attributed to the C-terminal HA-tag affecting MOV10 protein function.

3.3.3 MOV10 overexpression inhibits HIV-1 virus production and the infectivity of virions produced

The N-terminally and C-terminally-tagged MOV10 plasmids or relevant pGFP controls were also transfected into 293T cells together with pHIV-1_{NL4-3} to determine the effect of MOV10 overexpression on HIV-1 virus production and infectivity and, secondly, to ensure that the differential tags did not affect MOV10 protein function in the context of the full-length provirus. Virus production was quantified by $p24^{Gag}$ ELISA and consistent with the subgenomic constructs MOV10 overexpression decreased HIV-1 virus production by approximately 80%, 70% and 95% with myc-MOV10, MOV10-HA and T7-MOV10 overexpression, respectively, in comparison with the pGFP control (Figure 3.2C, left panel). Interestingly, the potency of inhibition was moderately

reduced in comparison with the subgenomic system, as ectopically overexpressed MOV10 inhibited VLP production more effectively than wild-type virus. Equal amounts of virus normalised by the $p24^{Gag}$ concentration was added to TZM-bl cells to measure the effect of MOV10 overexpression on the infectivity of virions produced. A dramatic decrease in virion infectivity was observed, whereby this was completely abolished with myc-MOV10 and T7-MOV10 overexpression and reduced by approximately 90% with MOV10-HA overexpression relative to the pGFP control (Figure 3.2C, right panel). Again, despite higher MOV10-HA expression levels (Figure 3.2A), its ability to inhibit HIV-1 virus production and infectivity was reduced in comparison with myc-MOV10 and T7-MOV10, and for this reason the pCMV4-MOV10-HA plasmid was not used for any further experiments. Therefore, overexpression of MOV10 inhibits HIV-1 virus production and the infectivity of virions produced.

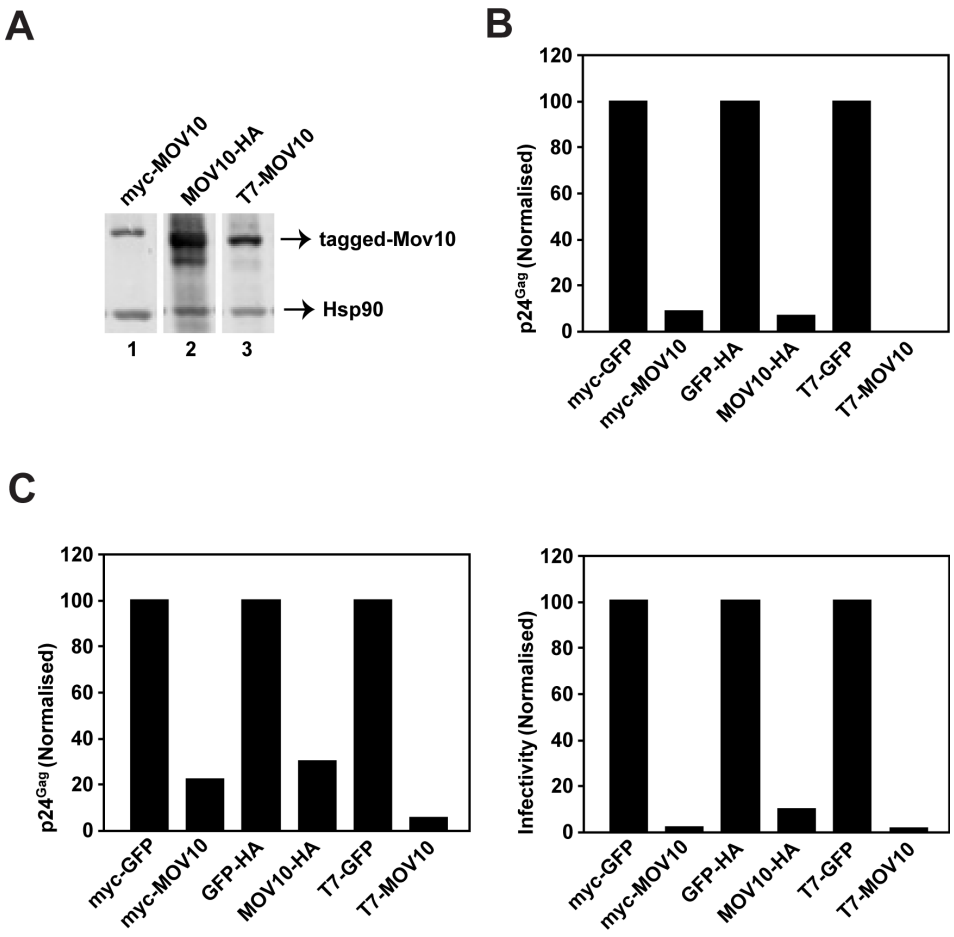


Figure 3.2. MOV10 overexpression inhibits HIV-1 virus production and the infectivity of virions produced.

(A) Expression of N-terminally and C-terminally tagged MOV10 proteins. The MOV10 cDNA was cloned into three expression plasmids with N-terminal and C-terminal tags to generate pcDNA3.1-myc-MOV10, pCMV4-MOV10-HA and pT7-MOV10. 293T cells were transfected with each of these plasmids and harvested approximately 24hrs post-transfection. Cells were lysed and analysed by immunoblotting with anti-MOV10 and anti-Hsp90 antibodies. **(B) Overexpression of MOV10 inhibits HIV-1 VLP production.** 293T cells were co-transfected with GPV-RRE (0.5 μg), pRev (0.25 μg) and MOV10 expressing plasmids described in panel (A) (1.5 μg) or pcDNA3.1-myc-GFP, pCMV4-GFP-HA and pT7-GFP (1.5 μg) as a control. VLPs were quantified by $p24^{Gag}$ ELISA as described in Figure 3.1B. **(C) MOV10 overexpression inhibits HIV-1 virus production and the infectivity of virions produced.** 293T cells were co-transfected with pHIV-1_{NL4-3} (0.5 μg) and MOV10 expressing plasmids described in panel (A) (1.5 μg) or plasmids expressing GFP controls as described in panel (B). Virus production was quantified by $p24^{Gag}$ ELISA and infectivity was measured by TZM-bl assay as described in Figure 3.1B. Results for panels (B) and (C) are normalised to the relevant GFP control, which is set at 100%.

3.4 MOV10 overexpression decreases HIV-1 virus production and the infectivity of virions produced in a dose-dependent manner

To test that MOV10-mediated inhibition of HIV-1 virus production and infectivity was not simply attributed to the ectopic overexpression of very high levels of MOV10, and also to test the potency of MOV10, pT7-MOV10 (pMOV10) was titrated into HeLa or 293T cells together with pHIV-1_{NL4-3}. The pT7-Luc (pLuc) control was also co-transfected with pMOV10 to ensure equivalent amounts of DNA in all transfections. Virus production was quantified by $p24^{Gag}$ ELISA and results showed that MOV10 overexpression decreased HIV-1 virion production in a concentration-dependent manner, whereby at the lowest pMOV10 concentration of 0.1 μg virus production was decreased by approximately 30% in HeLa and 293T cells, and at the highest pMOV10 amount of 1.5 μg this was reduced by approximately 70% and 80% in HeLa and 293T cells, respectively, compared to the pLuc control (Figure 3.3A). Cell lysates were also analysed by quantitative immunoblotting with an anti-$p24^{Gag}$ antibody, which detects precursor and processed Gag bands, to determine whether MOV10 overexpression affected Gag abundance or processing. Total cellular Gag levels were measured by quantifying all the Gag bands and at the maximum pMOV10 concentration Gag abundance was decreased by approximately 40% and 50% in HeLa and 293T cells, respectively, relative to the pLuc control (Figure 3.3A, compare lanes 1 and 7). Furthermore, Gag processing (total processed Gag bands divided by total Gag bands) was also reduced by approximately 10% and 40% in HeLa and 293T cells, respectively, in comparison to the pLuc control (Figure 3.3A, compare lanes 1 and 7).

Equal amounts of virus normalised by the $p24^{Gag}$ concentration was added to TZM-bl cells to determine the effect of MOV10 titration on virion infectivity. Results showed a dose-dependent decrease in HIV-1 virion infectivity with increasing concentrations of pMOV10, whereby at the lowest pMOV10 concentration of 0.1 μg the infectivity of virions produced was reduced by approximately 30% and 70% for HeLa and 293T cells, respectively, and at the highest pMOV10 concentration of 1.5 μg infectivity was decreased by approximately 90% for HeLa cells, and completely in the case of 293T cells compared to the pLuc control (Figure 3.3B). These data demonstrate that MOV10 overexpression decreases HIV-1 virus production and the infectivity of virions produced in a dose-dependent manner, and also reduces Gag expression and processing.

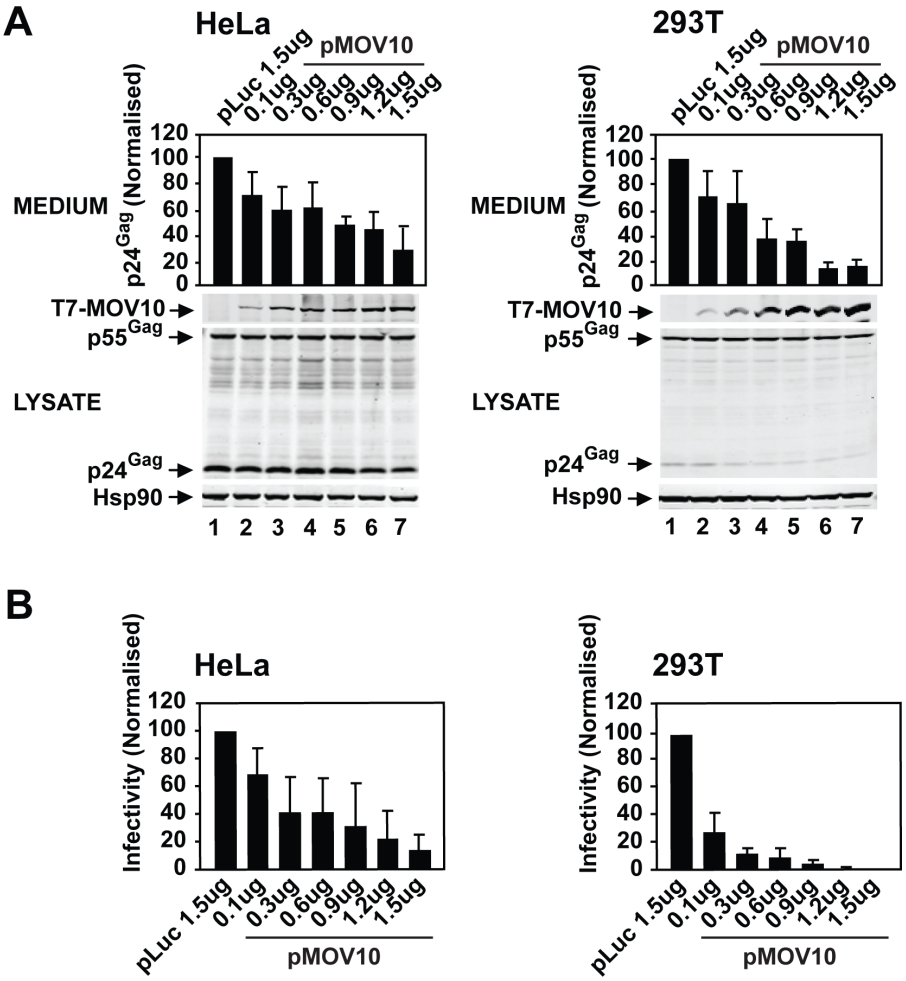


Figure 3.3. MOV10 overexpression decreases HIV-1 virus production and the infectivity of virions produced in a dose-dependent manner.

(A) MOV10 overexpression decreases HIV-1 virus production in a dose-dependent manner. HeLa or 293T cells were co-transfected with pHIV-1_{NL4-3} (0.5 μg) and pT7-MOV10 (pMOV10) at the indicated increasing concentrations or pT7-luciferase (Luc) as a control (pLuc). Virions were quantified by $p24^{Gag}$ ELISA as described in Figure 3.1B. Cells were lysed and analysed by immunoblotting with anti-T7, anti-$p24^{Gag}$ and anti-Hsp90 antibodies. **(B) Overexpression of MOV10 decreases the infectivity of HIV-1 virions produced in a concentration-dependent manner.** The infectivity of equal amounts of virus normalised by the $p24^{Gag}$ concentration from experiments depicted in panel (A) was measured by TZM-bl assay as described in Figure 3.1B. Results are normalised to the pLuc control, which is set at 100%. Values are the mean ± SD of 3 independent experiments.

3.5 Overexpression of P body and SG factors AGO2 and TIA-1 does not affect HIV-1 virus production or infectivity

The subcellular localisation of MOV10 is cytoplasmically diffuse and punctate with MOV10 detected in cytoplasmic P bodies and SGs (Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). AGO2 interacts with MOV10 and is also localised to P bodies, and similarly redistributes to SGs during cellular stress (Meister et al., 2005). TIA-1 is localised only to SGs and is essential for SG assembly (Kedersha et al., 1999; Gilks et al., 2004). A number of proteins that localise to P bodies and SGs have been implicated in the regulation of retroelements, including HIV-1 (chapter 1 sections 1.10.1 and 1.11.1). To determine whether the capacity to regulate HIV-1 replication is an inherent property of P body and SG proteins such as MOV10, the effect of AGO2 and TIA-1 overexpression on HIV-1 virus production and infectivity was determined.

293T cells were co-transfected with either pcDNA3.1-myc-MOV10 (pMOV10), pcDNA3.1-myc-AGO2 (pAGO2), pcDNA3.1-myc-TIA-1 (pTIA-1) or the pcDNA3.1-myc-GFP (pGFP), and cell lysates were analysed by immunoblotting with an anti-myc antibody to determine the relative expression levels of these proteins (Figure 3.4A). Subsequently, 293T cells were co-transfected with pHIV-1_{NL4-3} and either pMOV10, pAGO2, pTIA-1 or the pGFP control as described above. The effect on virion production was measured by $p24^{Gag}$ ELISA and results showed that unlike MOV10 overexpression, ectopic overexpression of AGO2 or TIA-1 did not decrease HIV-1 virion production (Figures 3.4B and C). In fact, TIA-1 overexpression moderately enhanced virus production (Figure 3.4C). The effect of these proteins on the infectivity of virions produced was also determined by TZM-bl assay and, similarly, both AGO2 and TIA-1 overexpression had no effect on HIV-1 infectivity either (Figures 3.4B and C). Therefore, although MOV10 overexpression inhibits HIV-1 virus production and infectivity, this is not an inherent function of all proteins that localise to P bodies and SGs.

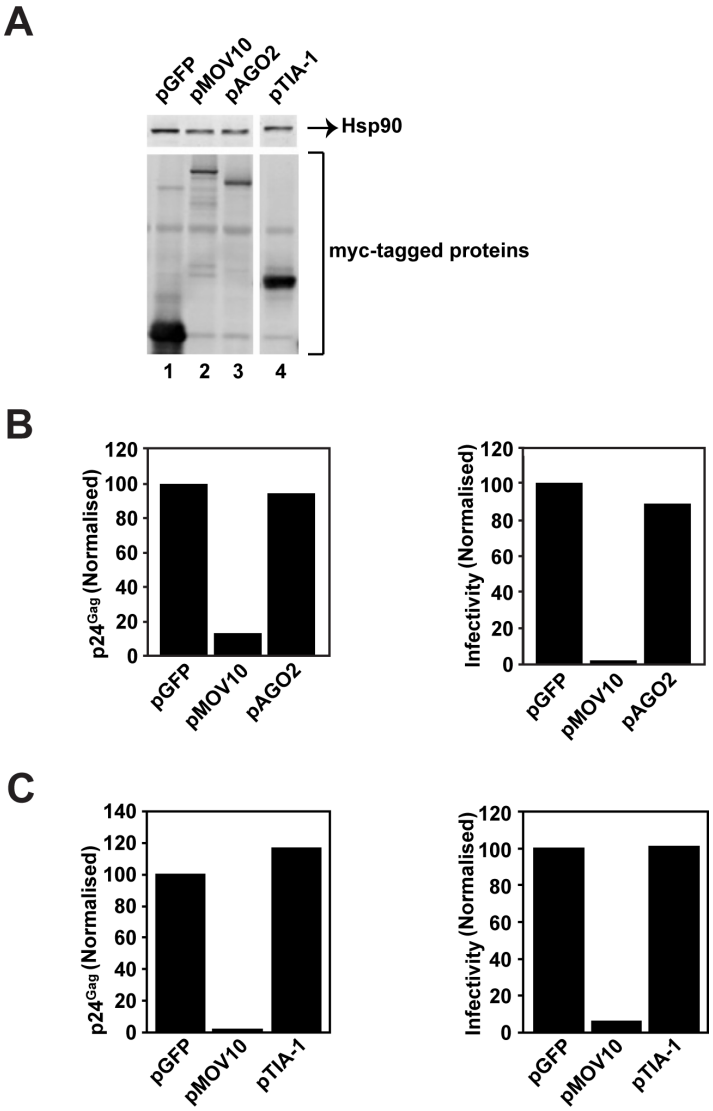


Figure 3.4. Overexpression of P body and SG factors AGO2 and TIA-1 does not affect HIV-1 virus production or infectivity.

(A) AGO2 and TIA-1 expression levels. 293T cells were co-transfected with either pcDNA3.1-myc-MOV10 (pMOV10), pcDNA3.1-myc-AGO2 (pAGO2), pcDNA3.1-myc-TIA-1 (pTIA-1) or the pcDNA3.1-myc-GFP (pGFP) control (1.5 μg). Cell lysates were analysed by immunoblotting with anti-myc and anti-Hsp90 antibodies. **(B) AGO2 overexpression has no effect on HIV-1 virus production or infectivity.** 293T cells were co-transfected with pHIV-1_{NL4-3} (0.5 μg) and either pMOV10, pAGO2 or the pGFP control (1.5 μg). Virus production was quantified by $p24^{Gag}$ ELISA and infectivity was measured by TZM-bl assay as described in Figure 3.1B. **(C) Overexpression of TIA-1 does not effect HIV-1 virus production or infectivity.** 293T cells were co-transfected with pHIV-1_{NL4-3} (0.5 μg) and either pMOV10, pTIA-1 or the pGFP control (1.5 μg). Virus production was quantified by $p24^{Gag}$ ELISA and infectivity was measured by TZM-bl assay as described in Figure 3.1B. Results are normalised to the pGFP control, which is set at 100%. Data are representative of 2 independent experiments.

3.6 Effect of MOV10 overexpression on other primate lentiviruses

3.6.1 SIVmac and HIV-1 vector system assay

As MOV10 overexpression potently inhibits HIV-1, the analysis of its antiviral activity was extended to the primate lentivirus SIVmac, which is a pathogenic SIV that arose as a result of infection of rhesus macaque monkeys with SIVsmm in US primate centres (Apetrei et al., 2005). Vector systems were adopted for these experiments; the SIVmac packaging plasmid (pSIV3+) encodes *gag*, *pro*, *pol* and all the regulatory and accessory proteins except *nef*, and also lacks *env* (Negre et al., 2000) (Figure 3.5A, top panel). The cis-acting element RRE is retained, however, the Psi is removed, and complete 5' R and U5 sequences are fused to the CMV promoter for transcription. A polyadenylation signal derived from the simian vacuolating virus 40 (SV40) replaces the 3' LTR. The SIVmac genome plasmid (pSIV-RMES4) is devoid of all viral proteins and expresses *GFP* from a CMV promoter (Negre et al., 2000) (Figure 3.5A, lower panel). The Psi, cPPT, RRE, and 3' LTR are intact, and the 5' R and U5 sequences are fused to a CMV promoter. A HIV-1 vector system was also tested as a control to ensure that results observed with the full-length provirus were reproducible with vectors. The HIV-1 packaging plasmid (p8.91) is similar to pSIV3+, however, it lacks all the accessory proteins and contains a polyadenylation signal derived from the insulin gene (Zufferey et al., 1997) (Figure 3.5B, top panel). The HIV-1 genome plasmid (pCSGW) is also similar to pSIV-RMES4, however GFP is expressed from a spleen focus-forming virus (SFFV) promoter, which is placed just upstream of a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) for enhanced expression (Bainbridge et al., 2001) (Figure 3.5B, lower panel). Also the 5' LTR is intact, however, this plasmid is a self-inactivating vector (SIN) and, therefore, the 3' LTR lacks the U3 region.

To produce SIVmac and HIV-1 vectors, 293T cells were co-transfected with either pSIV3+ and pSIV-RMES4 or p8.91 and pCSGW, respectively, together with a plasmid expressing the VSV glycoprotein (pVSV-G)(Fouchier et al., 1998) (Figure 3.5C). For MOV10 overexpression experiments, plasmids for SIVmac or HIV-1 vector production were co-transfected into 293T cells together with pT7-MOV10 (pMOV10) or pT7-Luc (pLuc) as a control. VSV-G pseudotyped particles incorporating a GFP-expressing genome were harvested and equal amounts of vector-containing supernatant was added to 293T cells. The effect of MOV10 overexpression on the production of infectious retrovirus particles was determined by measuring the percentage of GFP+ cells.

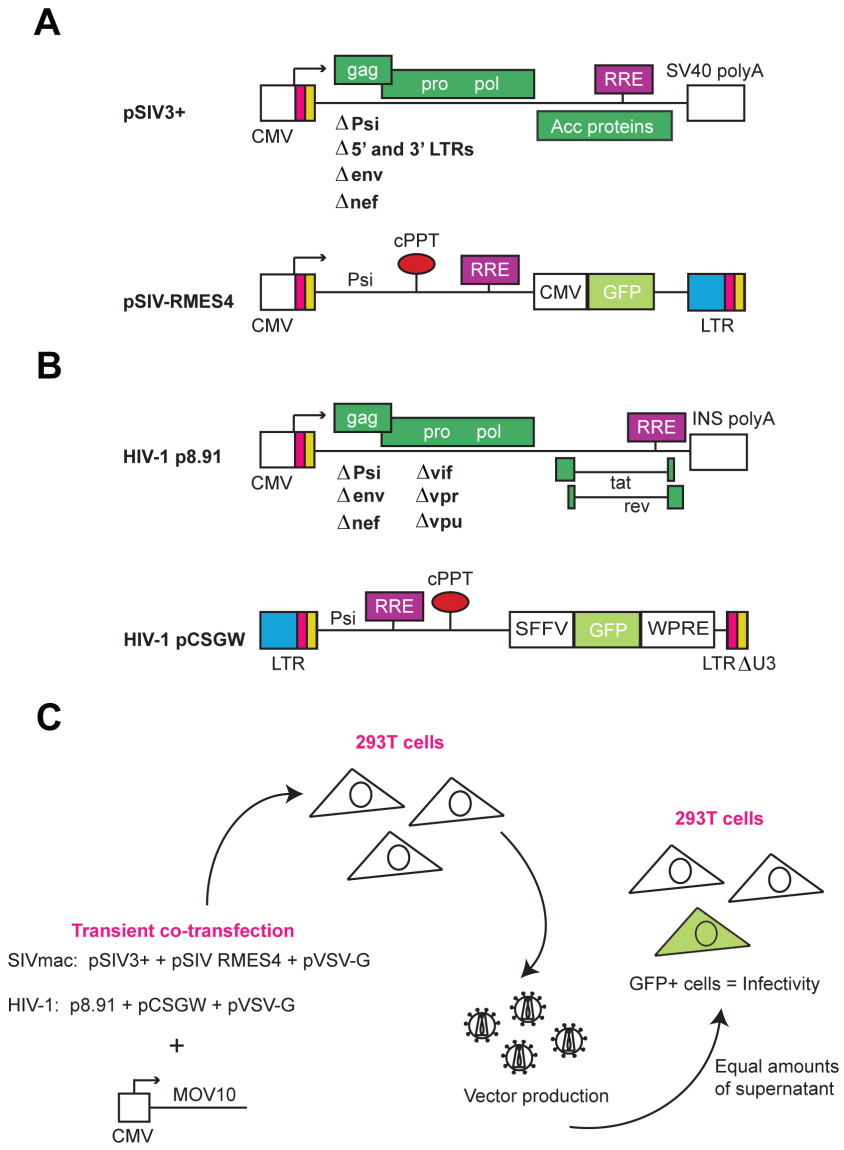


Figure 3.5. SIVmac and HIV-1 vector system assay.

(A) SIVmac vector system constructs. The SIVmac packaging plasmid, pSIV3+, encodes *gag*, *pro*, *pol*, all regulatory and accessory proteins and contains the RRE, however, lacks the Psi, *env* and *nef*. The U3 sequence is also deleted from the 5' LTR and replaced with the CMV promoter. The 3' LTR is entirely replaced with a polyadenylation signal from the simian vacuolating virus 40 (SV40). The SIVmac genome plasmid, pSIV-RMES4, is devoid of all viral proteins, and contains the Psi, cPPT and RRE. A CMV promoter drives expression of *GFP*. A CMV promoter also replaces the 5' LTR U3 sequence, however, the 3' LTR is intact. **(B) HIV-1 vector system constructs.** The HIV-1 packaging plasmid, p8.91, encodes *gag*, *pro*, *pol*, *tat*, *rev* and consists of the RRE, however, lacks all accessory proteins, *env* and the Psi. The U3 sequence in the 5' LTR is replaced with the CMV promoter, and the complete 3' LTR is replaced with a polyadenylation signal derived from the insulin gene (INS). The HIV-1 genome plasmid, pCSGW, lacks all viral proteins, and contains the Psi, RRE and cPPT. A spleen focus-forming virus (SFFV) promoter drives *GFP* expression, which is just upstream of a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). The 5' LTR is intact, however, this is a self-inactivating (SIN) vector and, therefore, lacks the U3 sequence in the 3' LTR. **(C) Vector system assay.** 293T cells were co-transfected with pSIV3+, pSIV-RMES4 and pVSV-G to produce SIVmac vectors, or p8.91, pCSGW and pVSV-G to produce HIV-1 vectors, together with pT7-MOV10 (pMOV10) or the pT7-Luc (pLuc) control. Vectors were harvested approximately 48hrs post-transfection and equal volumes of vector-containing supernatant was added to 293T cells. The effect on production of infectious vector particles was determined by quantifying the percentage of GFP+ 293T cells by fluorescence activated cell sorting (FACS).

3.6.2 MOV10 overexpression inhibits the production of infectious SIVmac and HIV-2 particles

The effect of MOV10 overexpression on the production of infectious SIVmac and HIV-1 vectors was tested as described, and similar to the observations for wild-type HIV-1, the production of both SIVmac and HIV-1 infectious particles was dramatically reduced by over 90% compared to the pLuc control (Figure 3.6A). The vector system assay does not differentiate between an inhibition in SIVmac vector production or infectivity, as determined for the wild-type HIV-1 experiments. Therefore, to assess whether MOV10 overexpression could decrease SIVmac particle production, 293T cells were co-transfected with a plasmid expressing a full-length SIVmac239 provirus (pSIVmac239) and pMOV10 at increasing concentrations or the pLuc control. HIV-2 was also included in the analysis for which 293T cells were co-transfected with a plasmid expressing the full-length HIV-2_{ROD} provirus (pHIV-2_{ROD}) together with pMOV10 or the pLuc control. Cell lysates and sucrose-cushion purified virions were analysed by immunoblotting using an anti-HIV-1 $p24^{Gag}$ antibody that recognises epitopes within SIVmac $p27^{Gag}$ and HIV-2 $p26^{Gag}$ (corresponds to CA in these viruses). Similar to wild-type HIV-1, MOV10 overexpression decreased SIVmac and HIV-2 virion production in a dose-dependent manner (Figure 3.6B). In the case of HIV-2, Gag expression was also notably reduced (Figure 3.6B, compare lanes 6 and 9), however, this could not be clearly determined for SIVmac due to variations in loading as determined by the Hsp90 loading control (Figure 3.6B, lanes 4 and 5).

The effect of MOV10 overexpression on the production of infectious HIV-2 virions was determined by adding equal volumes of virus-containing supernatant to TZM-bl cells, as the HIV-2 Tat protein also transactivates transcription from the HIV-1 LTR. Results showed a pMOV10 concentration-dependent decrease in the production of infectious HIV-2 virions, whereby at the highest pMOV10 concentration of 1 μg virions were no longer infectious (Figure 3.6C). The decrease in HIV-2 infectivity was more dramatic than that for HIV-1 at similar concentrations of pMOV10, however, this is likely attributed to the fact that HIV-2 virions were not normalised prior to their addition to TZM-bl cells. Nevertheless, factoring in the small decrease in HIV-2 virus production as determined by immunoblotting (Figure 3.6B), it seems likely that MOV10 overexpression inhibits the infectivity of HIV-2 virions as well.

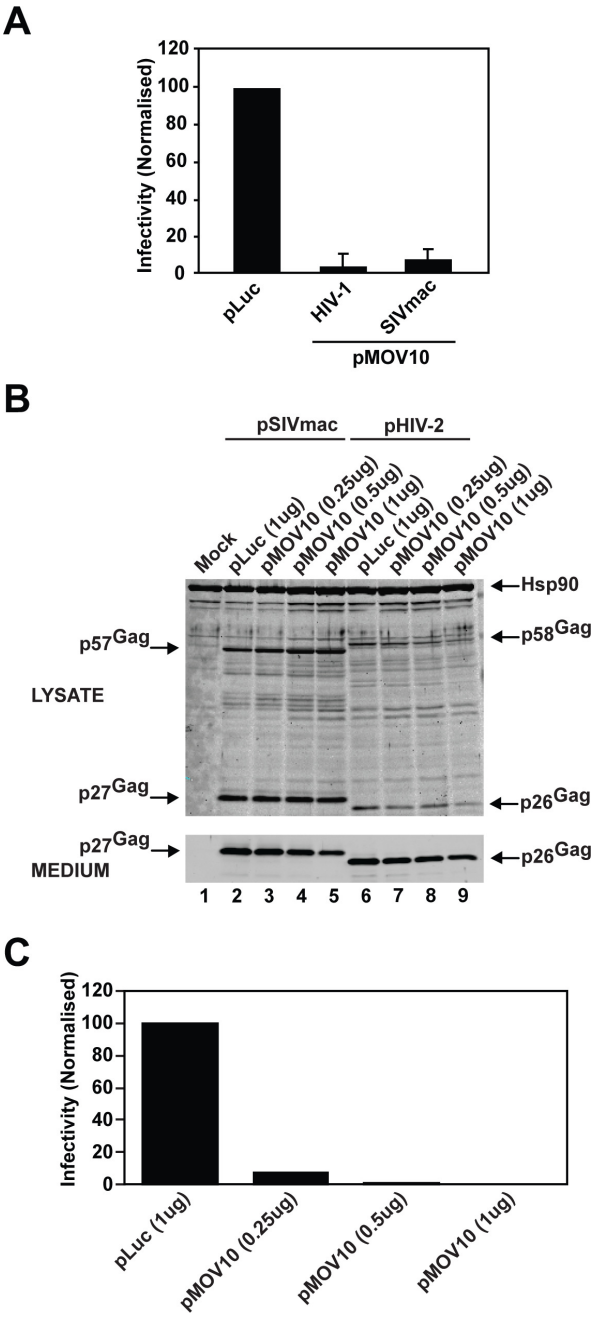


Figure 3.6. MOV10 overexpression inhibits the production of infectious SIVmac and HIV-2 particles.

(A) MOV10 overexpression inhibits the production of infectious SIVmac and HIV-1 vectors. 293T cells were co-transfected with p8.91 (1 µg), pCSGW (1 µg) and pVSV-G (0.5 µg) for HIV-1 vector production or pSIV3+ (1 µg), pSIV-RMES4 (1 µg) and pVSV-G (1 µg) for SIVmac vector production, together with pMOV10 or pLuc (0.5 µg). The effect on production of infectious vectors was determined by FACS as described in Figure 3.5C. **(B) MOV10 overexpression decreases SIVmac and HIV-2 virion production.** 293T cells were co-transfected with a full-length SIVmac provirus, pSIVmac239 (0.5 µg) or a full-length HIV-2_{ROD} provirus, pHIV-2_{ROD} (0.5 µg), together with pMOV10 at the indicated increasing concentrations or the pLuc control. Cell lysates and virions purified through a 20% sucrose cushion were analysed by immunoblotting with anti-HIV-1 $p24^{Gag}$ and anti-Hsp90 antibodies. **(C) Overexpression of MOV10 inhibits the production of infectious HIV-2 virions.** The effect on production of infectious virions was determined by TZM-bl assay by adding equal volumes of HIV-2 virus-containing supernatant from the experiment in panel (B) to TZM-bl reporter cells. Results for panel (A) and (C) are normalised to the pLuc control, which is set at 100%. For panel (A) a single control bar set at 100% is graphed for simplicity and these values are the mean ± SD of 3 independent experiments.

3.7 Effect of MOV10 overexpression on retroviruses from other genera

3.7.1 MOV10 overexpression abolishes the production of infectious MLV virions

As MOV10 overexpression potently inhibits members of the primate lentivirus family the capacity of this protein to restrict retroviruses from within other genera was investigated. MLV is a gammaretrovirus, which belongs to the group of simple retroviruses that encode only *gag*, *pro*, *pol* and *env*. The effect of MOV10 ectopic overexpression on the production of infectious MLV particles was determined by co-transfecting 293T cells with a plasmid expressing the full-length MLV provirus (pMLV) (Yueh and Goff, 2003) and a surrogate genome expressing the HIV-1 Tat protein (pMLV-Tat) (Martin-Serrano et al., 2003), together with pVSV-G and either pT7-MOV10 (pMOV10) or the pT7-Luc (pLuc) control (Figure 3.7A). VSV-G pseudotyped MLV virions were harvested and equal amounts of virus-containing supernatant was added to TZM-bl reporter cells. Remarkably, MOV10 overexpression abolished the production of infectious MLV virions completely (Figure 3.7B). Again, as MLV virions were not normalised prior to their infection of TZM-bl cells, the effect of MOV10 overexpression on MLV virion production and infectivity could not be differentiated. Therefore, ectopic overexpression of MOV10 not only restricts primate lentiviruses, but also inhibits the production of infectious MLV particles.

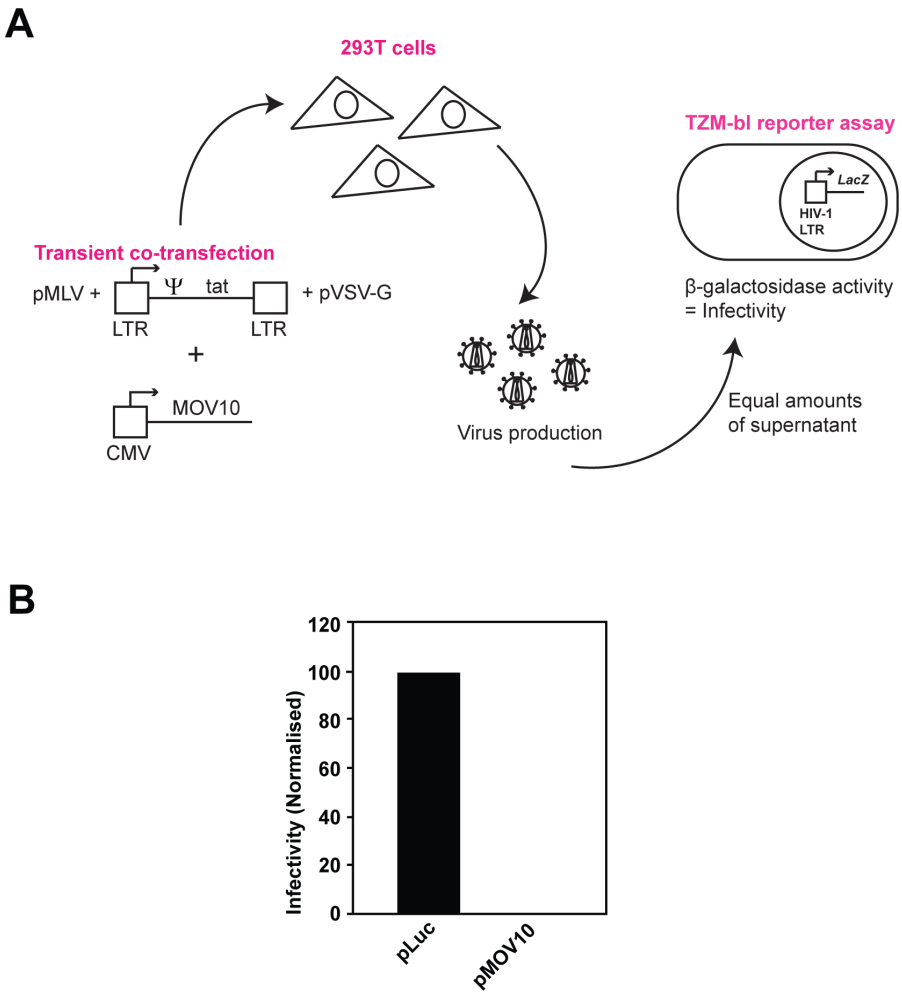


Figure 3.7. MOV10 overexpression abolishes the production of infectious MLV virions.

(A) MLV assay. 293T cells were co-transfected with a plasmid expressing the full-length MLV provirus, pMLV, a surrogate MLV plasmid expressing the HIV-1 Tat protein, pMLV-Tat, and pVSV-G together with pT7-MOV10 (pMOV10) or the pT7-Luc (pLuc) control. Approximately 48hrs post-transfection, MLV virions were harvested and equal volumes of virus-containing supernatant was added to TZM-bl cells to determine the effect on production of infectious virions. **(B) MOV10 overexpression abolishes the production of infectious MLV virions.** 293T cells were co-transfected with pMLV (0.2 μg), pMLV-Tat (0.2 μg), pVSV-G (0.1 μg) and either pMOV10 or the pLuc control (0.5 μg). The effect on production of infectious virions was determined as described in panel (A). Results are normalised to the pLuc control, which is set at 100%. Values are the mean ± SD of 3 independent experiments.

3.7.2 Production of infectious M-PMV virions is restricted by MOV10 overexpression

M-PMV belongs to the betaretrovirus genera of the retrovirus family and is also a simple retrovirus that encodes only *gag*, *pro*, *pol* and *env*. To determine the effect of MOV10 overexpression on the production of infectious M-PMV, a plasmid expressing the full-length M-PMV provirus in which *env* is replaced with HIV-1 *tat* (pMTΔE) (Doehle et al., 2006) was co-transfected into 293T cells together with pVSV-G and either pT7-MOV10 (pMOV10) or the pT7-Luc (pLuc) control (Figures 3.8A and B). VSV-G pseudotyped M-PMV virions expressing HIV-1 Tat were harvested and equal amounts of virus-containing supernatant was added to TZM-bl cells to establish the effect of MOV10 overexpression on production of infectious particles (Figure 3.8B). Similar to previous results with HIV-1, HIV-2, SIVmac and MLV, the production of infectious M-PMV virions was dramatically reduced by over 95% relative to the pLuc control (Figure 3.8C). Cell lysates and sucrose-cushion purified virions were also analysed by immunoblotting using an antibody that recognises an epitope within M-PMV $p27^{Gag}$ (corresponds to CA), which detects both precursor and processed Gag bands. As for HIV-1, HIV-2 and SIVmac, MOV10 overexpression decreased the production of M-PMV virions and markedly reduced cellular Gag expression in a pMOV10 concentration-dependent manner (Figure 3.8D, compare lanes 1 and 3). Together, these data support a potential antiviral role for MOV10 in the replication of simple and complex retroviruses.

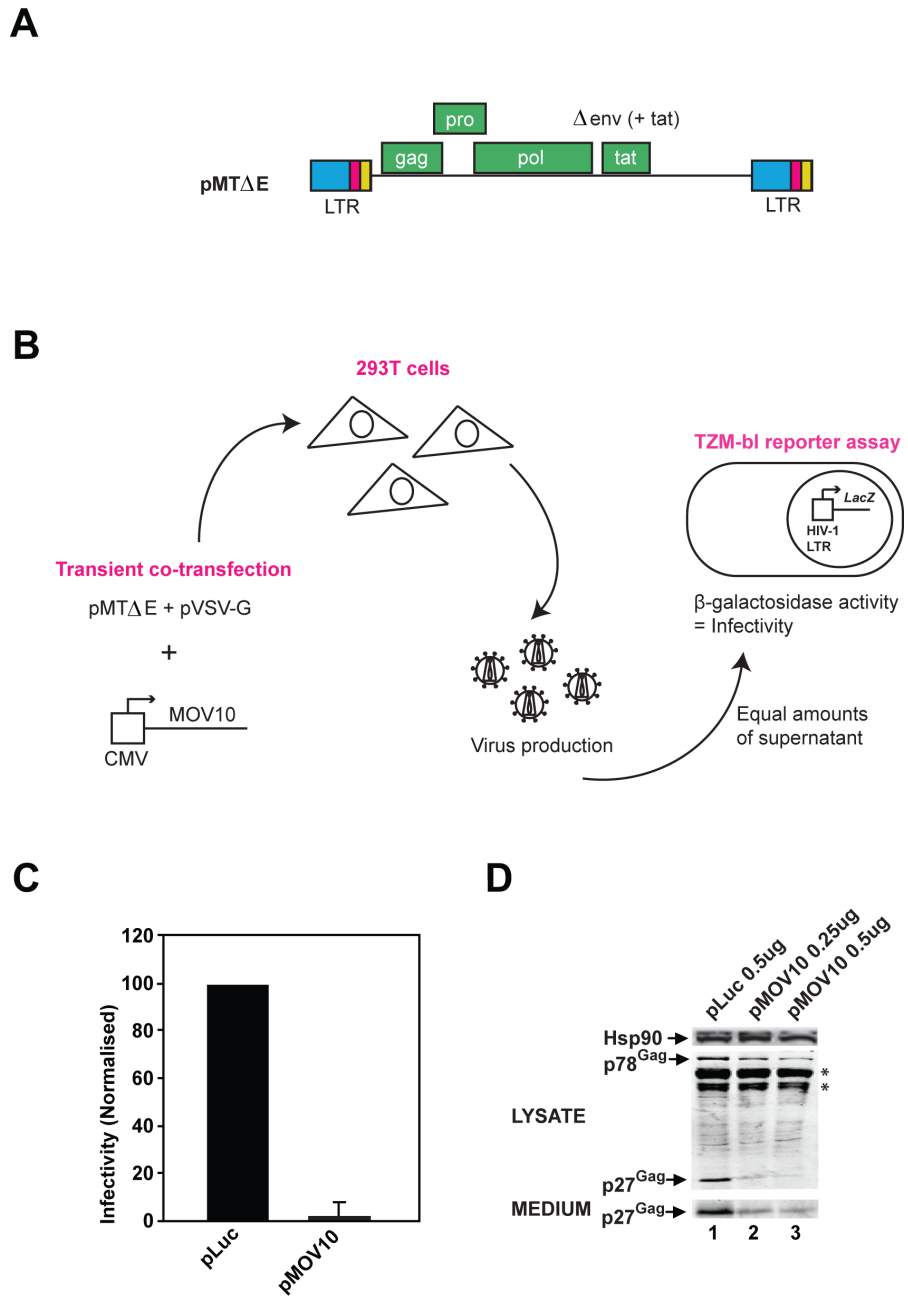


Figure 3.8. Production of infectious M-PMV virions is restricted by MOV10 overexpression.

(A) M-PMV proviral construct (pMTΔE). pMTΔE expresses a full-length M-PMV provirus in which *env* is replaced with HIV-1 *tat*. **(B) M-PMV assay.** 293T cells were co-transfected with pMTΔE and pVSV-G together with pT7-MOV10 (pMOV10) or the pT7-Luc (pLuc) control. Approximately 48hrs post-transfection, M-PMV virions were harvested and the effect on production of infectious virions was determined by TZM-bl assay by adding equal volumes of virus-containing supernatant to the TZM-bl reporter cell line. **(C) Production of infectious M-PMV virions is restricted by MOV10 overexpression.** 293T cells were co-transfected with pMTΔE (1 µg) and pVSV-G (0.5 µg) together with pMOV10 or the pLuc control (0.5 µg). The effect on production of infectious virions was determined as described in panel (B). **(D) MOV10 overexpression decreases M-PMV virion production.** Cell lysates and M-PMV virions purified through a 20% sucrose cushion from the experiments in panel (C) were analysed by immunoblotting with anti-M-PMV $p27^{Gag}$ and anti-Hsp90 antibodies (* refers to non-specific bands). Results for panel (C) are normalised to the pLuc control, which is set at 100% and these values are the mean ± SD of 3 independent experiments. The data in panel (D) is representative of 2 independent experiments.

3.8 Effect of MOV10 overexpression on endogenous retroelements

3.8.1 Retrotransposition assay

MOV10 overexpression restricts retroviruses from the lentivirus, gammaretrovirus and betaretrovirus subfamilies and, therefore, displays broad antiretroviral activity. The genome organisation of ERVs is similar to that of exogenous retroviruses, although these have an intracellular life cycle partially owing to the lack of a functional *env* gene, and have also entered the germ line allowing them to be vertically transmitted (chapter 1 section 1.6). On the other hand, retrotransposons are endogenous retroelements with distinct genome organisation from retroviruses and furthermore, retrotransposons couple the process of reverse transcription and integration through a mechanism called TPRT that takes place in the nucleus (chapter 1 section 1.7.1). Considering the broad antiretroviral effect of MOV10 overexpression, as well as the essential roles of MOV10 homologs in the suppression of endogenous retroelements (Frost et al., 2010; Olivieri et al., 2010; Zheng et al., 2010) (chapter 1 section 1.13.2), the capacity of MOV10 to regulate ERVs and retrotransposons was investigated. The effect of MOV10 ectopic overexpression on the replication of the highly active mouse LTR-containing ERV IAP, and the human non-LTR autonomous LINE-1 and non-autonomous Alu retrotransposons was determined.

Established cell-culture based retrotransposition assays were adopted to test this (Boeke et al., 1985; Moran et al., 1996; Esnault et al., 2002). Plasmids expressing IAP (pGL3-IAP92L23neo^{TNF}) (Dewannieux et al., 2004), LINE-1 (pJM101/L1.3) (Moran et al., 1999) and Alu (pAlu-neo^{Tet}) (Dewannieux et al., 2003), all of which contain an antisense neomycin resistance gene cassette (*neo*) at the 3' end driven by its own promoter and disrupted by an intron, were used to test this (Figure 3.9). Expression of *neo* occurs only after a complete retrotransposition cycle: specifically, transcription of the retroelement RNA, splicing of the intron in the *neo* cassette, translation of proteins, reverse transcription and integration of the retroelement cDNA into the host chromosomal DNA. The retrotransposition frequency was established by counting G418-resistant colonies.

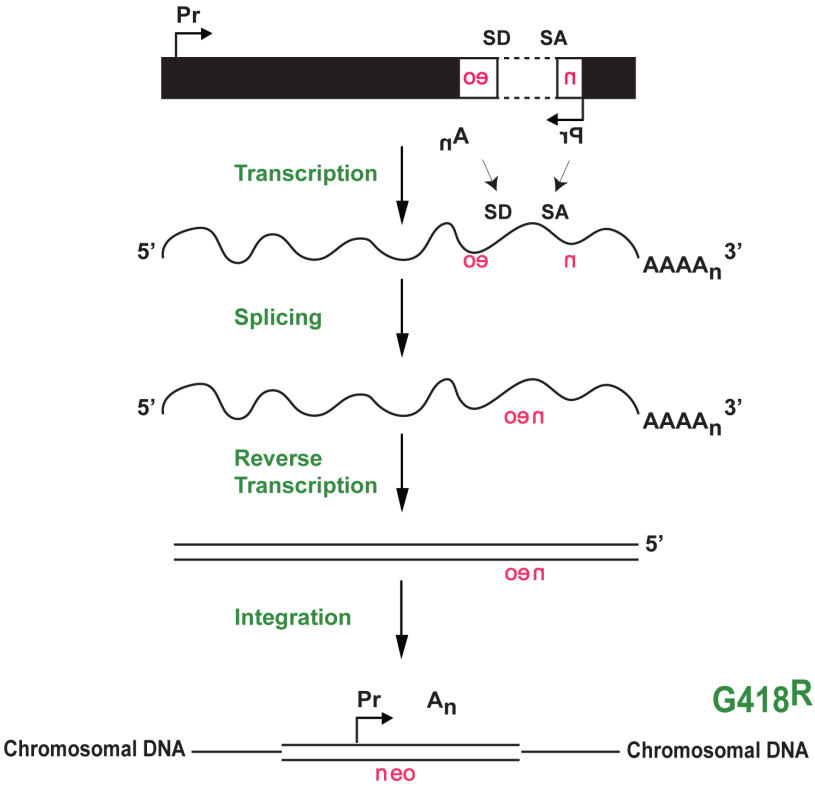


Figure 3.9. Retrotransposition assay.

The IAP, LINE-1 and Alu cDNA contains an antisense neomycin resistance gene cassette (*neo*) at the 3' end disrupted by an intron and driven by its own promoter. *Neo* expression occurs only after transcription of the RNA, splicing out of the intron, reverse transcription and integration of the cDNA into the host cell genome. The retrotransposition frequency was determined by G418 selection of the cultures and counting the G418-resistant colonies.

3.8.2 MOV10 overexpression suppresses the retrotransposition of LTR and non-LTR endogenous retroelements

HeLa cells were co-transfected with pGL3-IAP92L23neo^{TNF} (pIAP), pJM101/L1.3 (pLINE-1) or pAlu-neo^{Tet} (pAlu) together with either pT7-MOV10 (pMOV10) or the pT7-Luc (pLuc) control. For Alu retrotransposition assays cells were also co-transfected with a plasmid expressing LINE-1 ORF2p (pORF2p) as Alu elements do not encode any proteins and depend on LINE-1 proteins for their retrotransposition. MOV10 overexpression drastically reduced the number of G418-resistant cell colonies for all endogenous retroelements tested, with almost no colonies detectable for Alu and IAP, and over a 95% decrease in colonies for LINE-1 relative to the pLuc control (Figure 3.10A). Therefore, ectopic overexpression of MOV10 potently restricts the retrotransposition of the mouse ERV IAP and human retrotransposons LINE-1 and Alu.

To ensure that overexpression of MOV10 had no direct effect on *neo* expression or selection, HeLa cells were co-transfected with a pcDNA3.1 plasmid containing a *neo* cassette (pcDNA3.1-*neo*) together with pT7-MOV10 (pMOV10) or the pT7-Luc (pLuc) control. A similar number of colonies were counted with MOV10 overexpression as the pLuc control suggesting that MOV10 does not directly hinder *neo* expression or selection, and specifically suppresses the propagation of LTR and non-LTR endogenous retroelements (Figure 3.10B). Therefore, MOV10 overexpression restricts the replication of genetically distinct exogenous and endogenous retroelements.

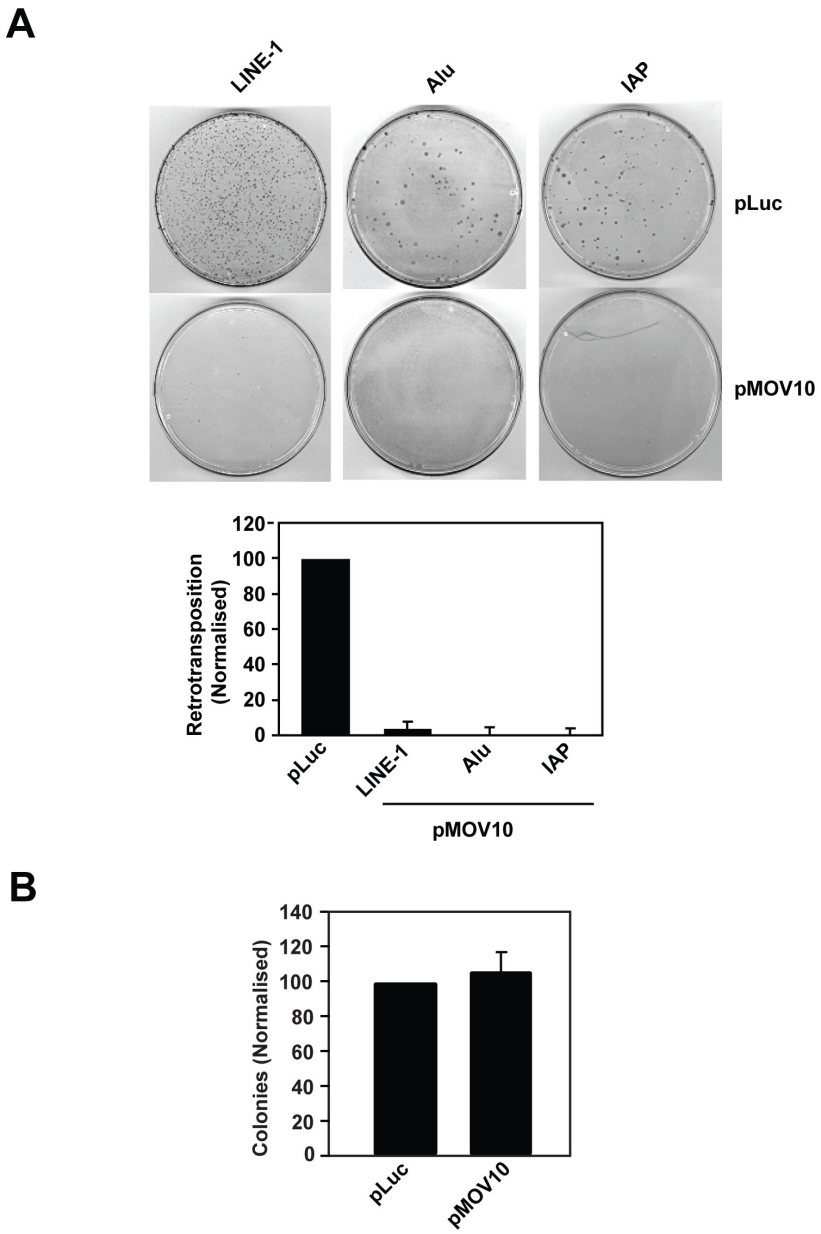


Figure 3.10. MOV10 overexpression suppresses the retrotransposition of LTR and non-LTR endogenous retroelements.

(A) MOV10 overexpression suppresses the retrotransposition of LTR and non-LTR endogenous retroelements. HeLa cells were co-transfected with plasmids expressing the IAP (pGL3-IAP92L23neo^{TNF}) (1.5 μg), LINE-1 (pJM101/L1.3) (1.5 μg) or Alu (pAlu-neo^{Tet}) (1 μg) cDNAs containing the *neo* cassette as described in Figure 3.9, together with pT7-MOV10 (pMOV10) or the pT7-Luc (pLuc) control (1 μg). For Alu retrotransposition assays, HeLa cells were also co-transfected with a plasmid expressing LINE-1 ORF2p (pORF2p) (0.5 μg). The retrotransposition frequency was determined by G418 selection of the cultures as described in Figure 3.9. **(B) MOV10 overexpression has no direct effect on *neo* expression or selection.** HeLa cells were co-transfected with a pcDNA3.1 plasmid containing a *neo* cassette (pcDNA3.1-*neo*) (0.3 μg) together with pMOV10 or the pLuc control (1 μg). The retrotransposition frequency was determined by G418 selection of the cultures as described in Figure 3.9. Results are normalised to the pLuc control, which is set at 100%. For panel (A) a single control bar set at 100% is graphed for simplicity. Values are the mean ± SD of 3 independent experiments.

3.9 Discussion

The interactions and associations of MOV10 with antiviral proteins and pathways implicated in the regulation of retroviruses and retrotransposons (Meister et al., 2005; Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008) (chapter 1 sections 1.10.1, 1.11.1 and 1.12.5), as well as the essential role of MOV10 homologs Armitage and MOV10L1 in the suppression of ERVs and retrotransposons (Frost et al., 2010; Olivieri et al., 2010; Zheng et al., 2010) (chapter 1 section 1.13.2) makes MOV10 a credible candidate in the search for novel anti-retroelement host factors. The results presented in this chapter reveal that MOV10 overexpression potently inhibits the production and infectivity of a diverse panel of exogenous retroviruses, as well as a highly active mouse ERV, and also effectively suppresses the retrotransposition of human non-LTR retrotransposons. These data support a potential inhibitory role for MOV10 in the retroelement life cycle.

Ectopically overexpressed MOV10 decreases the production of HIV-1, HIV-2, SIVmac and M-PMV virions in a dose-dependent manner (Figures 3.3A, 3.6B and 3.8D). The overall decrease in cellular Gag abundance may account for this observation and, furthermore, as virion assembly is a cooperative process a reduction in intracellular Gag expression may impact plasma membrane targeting and, therefore, Gag processing (Hatziioannou et al., 2005). This seems to be true for HIV-1 virion production in 293T cells whereby at the maximum concentration of pMOV10 both cellular Gag levels and processing are reduced (Figure 3.3A, right panel). However, for HIV-1 virion production in HeLa cells although the percentage decrease in cellular Gag expression is equivalent to that observed in 293T cells, the effect on Gag processing is minimal (Figure 3.3A, left panel), implying that plasma membrane targeting is mostly normal. Alternatively, Gag targeting to the plasma membrane may not be impacted at all and for virus produced in 293T cells, MOV10 overexpression may directly affect processing and, therefore, virion maturation. These differential observations may underlie the more potent inhibition of HIV-1 virus production in 293T cells compared to HeLa cells at higher concentrations of MOV10 (Figure 3.3A). Furthermore, this disparity between cell types may be attributed to higher MOV10 expression levels in 293T cells owing to the presence of the SV40 large T antigen, which induces DNA replication from the SV40 origin of replication in a plasmid. HIV-2 and M-PMV cellular Gag abundance is

also notably decreased suggesting that this common mode of action similarly impacts multiple retroviruses (Figures 3.6B and 3.8D).

Three groups have now assessed the role of MOV10 in HIV-1 replication (Burdick et al., 2010; Furtak et al., 2010; Wang et al., 2010). Furtak et al and Burdick et al similarly reported a dose-dependent decrease in HIV-1 virion production with MOV10 overexpression (Burdick et al., 2010; Furtak et al., 2010) and, furthermore, the latter group also detected a MOV10 concentration-dependent decrease in Gag expression and efficiency of Gag processing (Burdick et al., 2010). In contrast, Wang et al observed no effect of MOV10 overexpression on HIV-1 Gag processing (Wang et al., 2010), a difference that may be at least partially explained by lower MOV10 expression levels in producer cells. It would be important to determine whether the reduction in cellular Gag abundance is an outcome of decreased Gag translation or increased Gag turnover, or possibly an alternative mechanism.

Interestingly, MOV10 overexpression inhibits the production of VLPs more effectively than wild-type virions. VLPs are produced from the GPV-RRE subgenomic construct, which encodes only complete *gag*, *pro*, *pol* and *vif* genes (Figure 3.1A). Therefore, more effective inhibition of VLPs may be attributed to the lack of a viral accessory factor that can directly or indirectly counteract MOV10 during wild-type infection. Transfection of a plasmid expressing the HIV-1 regulatory protein Tat in trans does not antagonise MOV10-mediated inhibition of VLP production, excluding Tat as the missing factor responsible (data not shown). Although the percentage difference by which MOV10 overexpression inhibits VLPs and wild-type virions is a moderate 5-15% (Figures 3.2B and C), in the context of endogenous levels of MOV10 and physiological infection the presence of a viral accessory protein able to counteract MOV10 could make the difference between inefficient and efficient viral spread and replication.

Overexpression of MOV10 in producer cells also drastically decreases the infectivity of HIV-1 virions produced, the inhibition of which is more potent than that observed for virion production (Figure 3.3B). Additionally, MOV10 overexpression also restricts the production of infectious SIVmac, HIV-2, MLV and M-PMV particles (Figures 3.6A and C, 3.7B and 3.8C). The production of HIV-2 infectious particles is abolished at the maximum pMOV10 concentration, however, the effect on virion production is only

moderate (Figures 3.6B and C). Therefore, defective HIV-2 virion production does not completely account for the potent decrease in virion infectivity. Alternatively, M-PMV cellular Gag expression and virion production are greatly reduced in comparison with HIV-1 and HIV-2 at similar concentrations of pMOV10 (Figure 3.8D). For this reason it is difficult to differentiate the effect of MOV10 overexpression on M-PMV virion production or infectivity. These results agree with those reported by other groups, whereby overexpression of human MOV10 inhibited HIV-1, SIVmac, African green monkey derived SIV (SIVagm), feline immunodeficiency virus (FIV), EIAV and MLV (Burdick et al., 2010; Furtak et al., 2010; Wang et al., 2010). Moreover, Furtak et al and Burdick et al also observed a stronger inhibition of HIV-1 virion infectivity compared to virion production (Burdick et al., 2010; Furtak et al., 2010). In contrast, overexpression of MOV10 in target cells has no effect on HIV-1 infectivity (Burdick et al., 2010; Wang et al., 2010).

MOV10 is a P body component that shifts to SGs under conditions of cellular stress (Gallois-Montbrun et al., 2007). The role of P body and SG factors in the life cycle of retroviruses, and especially HIV-1, has been controversial whereby both inhibitory and stimulatory functions have been proposed (chapter 1 sections 1.10.1 and 1.11.1). Reed et al proposed a role for the P body protein DDX6 in HIV-1 Gag assembly (Reed et al., 2012). In contrast, Chable-Bessia et al reported the silencing of P body components DDX6, GW182, Lsm1 and XRN1 to facilitate HIV-1 infection (Chable-Bessia et al., 2009). Furthermore, the intrinsic HIV-1 restriction factors A3F and A3G co-localise with MOV10 in P bodies and SGs (Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). However, Phalora et al recently confirmed no effect of P body depletion through knockdown of DDX6 and Lsm1 on HIV-1 replication, incorporation of APOBEC3 proteins into virions or APOBEC3-mediated restriction of HIV-1 infection (Phalora et al., 2012). Consistent with these latter findings, the results presented in this chapter demonstrate that the capacity to regulate HIV-1 is not inherent to all P body and SG proteins. Overexpression of the P body and SG component AGO2, which interacts with MOV10 and members of the APOBEC3 family (Meister et al., 2005; Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008), has no effect on HIV-1 virus production or the infectivity of virions produced (Figure 3.4B). Likewise, overexpression of the essential SG protein TIA-1, at a concentration that was observed to induce SGs (data not shown), did not decrease HIV-1 virus production or infectivity

either, and actually increased virion production moderately (Figure 3.4C). Similarly, Wang et al showed the overexpression of P body components AGO1, AGO2 and DDX3 to have no effect on HIV-1 infectivity, and Burdick et al reported comparable results for DCP1a and DCP2 overexpression (Burdick et al., 2010; Wang et al., 2010). Therefore, individual proteins, and likely their cellular functions, as opposed to the association of these factors with P bodies determines their capacity to regulate HIV-1 replication, and similar detailed analyses will be required to understand the relationship between SGs and HIV-1 infection.

The MOV10 ortholog Armitage in *Drosophila melanogaster* and mammalian paralog MOV10L1 are both essential for piRNA-guided suppression of endogenous retroelements (Olivieri et al., 2010) Frost, 2010 #77}(Zheng et al., 2010) (chapter 1 section 1.13.2). Armitage is necessary for PIWI protein function, which mediates PTGS of endogenous retroelements in both the germ line and somatic cells (Olivieri et al., 2010). Alternatively, MOV10L1 is required for the function of mouse PIWI proteins, which mediate TGS of ERVs and retrotransposons, such as IAP and LINE-1, in the germ line of male mice through DNA methylation (Frost et al., 2010; Zheng et al., 2010). Interestingly, ectopic overexpression of human MOV10 suppresses the retrotransposition of genetically diverse IAP, LINE-1 and Alu endogenous retroelements implying that the capacity of MOV10 to restrict ERVs and retrotransposons is evolutionarily conserved (Figure 3.10A). A recent study similarly showed MOV10 overexpression to inhibit IAP retrotransposition (Lu et al., 2012).

Interestingly, human LINE-1 derived endo-siRNAs have been reported to restrict LINE-1 retrotransposition by an RNAi mechanism (Yang and Kazazian, 2006) and, more recently, natural occurring endo-siRNAs have been proposed to transcriptionally silence LINE-1 expression through DNA methylation of the promoter (Chen et al., 2012). Furthermore, knockdown of DICER-1 enhances LINE-1 retrotransposition (Yang and Kazazian, 2006). The role of P bodies and SGs in the replication of ERVs and retrotransposition remains to be established, although individual P body and SG factors have been implicated in the regulation of endogenous retroelements. The APOBEC3 family of proteins restrict the retrotransposition of LINE-1 and Alu retrotransposons (chapter 1 section 1.8.2.10), and knockdown of P body proteins DDX6 and eIF4E-T enhances IAP retrotransposition (Lu et al., 2011). Furthermore, LINE-1 ORF1p has

been reported to co-localise with other SG factors in SGs (Goodier et al., 2007). The mechanism by which MOV10 overexpression suppresses IAP, LINE-1 and Alu retrotransposition is not yet clear, and may be dependent on its known cellular associations with small RNA-mediated post-transcriptional RNA silencing pathways, P bodies and SGs and/or the APOBEC3 proteins. However, Lu et al recently demonstrated P bodies to be dispensable for the restriction of IAP replication by ectopically overexpressed MOV10 (Lu et al., 2012).

CHAPTER 4

RESULTS

MOV10 overexpression restricts the replication of retroelements: mechanism and structure-function analysis

4.1 Introduction

The results presented in chapter 3 demonstrate that MOV10 overexpression restricts the replication of a diverse panel of retroviruses and retrotransposons. For exogenous retroviruses this inhibitory activity has dual aspects, whereby ectopic overexpression of MOV10 decreases cellular Gag expression and virion production, and also potently reduces the infectivity of virions produced. In this chapter, the localisation of MOV10 and HIV-1 Gag as well as analysis of domains within HIV-1 Gag that may be necessary for MOV10-mediated reduction in virion production were explored to begin to dissect the mechanism by which MOV10 affects the production of retroviral particles. The mechanism by which MOV10 overexpression decreases the infectivity of retroviral particles produced is unclear. As MOV10 is a putative RNA helicase, the effect of MOV10 ectopic overexpression on the incorporation of HIV-1 viral RNA into nascent virions was determined. Furthermore, the interaction of MOV10 with antiviral proteins A3F and A3G (Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008) questions whether MOV10 may function by impacting the process of reverse transcription. Lastly, the dependence of MOV10 anti-retroelement activity on its putative RNA helicase motifs was investigated through structure-function studies.

4.2 Ectopically overexpressed MOV10 and HIV-1 Gag co-localise in SG-like cytoplasmic domains

MOV10 overexpression decreases the production of HIV-1 virions, which may be caused by the reduction in cellular Gag abundance. The subcellular localisation of ectopically overexpressed MOV10 and HIV-1 Gag was determined to loosely assess whether MOV10 may mediate this effect through an association with Gag. HeLa cells were co-transfected with GPV-RRE and pRev, together with pcDNA3.1-myc-MOV10 (pMOV10). Cells were fixed in 4% paraformaldehyde (PFA) and analysed by immunofluorescence using anti-myc, anti-$p17^{Gag}$ ($p17^{Gag}$ corresponds to MA) and anti-$p24^{Gag}$ antibodies. Cells were also stained with 4',6-diamidino-2-phenylindole (DAPI) which is a fluorescent dye that binds to DNA highlighting the cell nucleus. Remarkably, MOV10 and HIV-1 Gag strongly co-localised in cytoplasmic aggregates closely resembling SGs (Figure 4.1), implying that MOV10 may decrease HIV-1 virion production through a direct association with Gag in SG-like cytoplasmic domains.

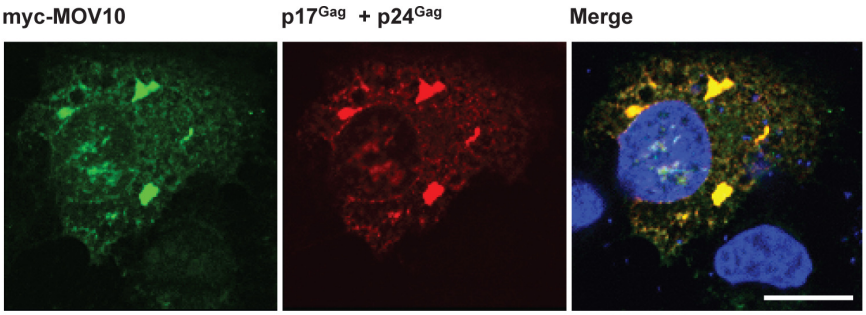


Figure 4.1. Ectopically overexpressed MOV10 and HIV-1 Gag co-localise in SG-like cytoplasmic domains.

HeLa cells were plated onto coverslips and co-transfected with GPV-RRE (1 µg) and pRev (0.25 µg), together with pcDNA3.1-myc-MOV10 (pMOV10) (0.5 µg). Approximately 24hrs post-transfection cells were fixed in 4% paraformaldehyde (PFA) and stained with anti-myc, anti-$p17^{Gag}$ and anti-$p24^{Gag}$ primary antibodies and appropriate Alexa Fluor conjugated secondary antibodies, as well as the 4',6-diamidino-2-phenylindole (DAPI) fluorescent dye to stain the nucleus. Coverslips were mounted onto slides and analysed by immunofluorescence. Scale bar = 10µm.

4.3 Gag determinants and MOV10 antiviral activity

4.3.1 GPV-RRE constructs

Co-localisation of ectopically overexpressed MOV10 and HIV-1 Gag indicates that MOV10 may directly or indirectly interact with Gag and/or interfere with Gag functions to mediate the decrease in cellular Gag expression and, therefore, potentially virion production. GPV-RRE NC (ΔNC+Z_t) and MA (ΔMA+M-src) domain mutants were tested to determine whether MOV10 overexpression requires these Gag domains for the reduction in virion production (Figure 4.2A). ΔMA+M-src lacks the MA domain, however, contains a heterologous myristylation sequence derived from the *c-src* gene for efficient plasma membrane targeting. Similarly, ΔNC+Z_t is deleted of the NC region and this is replaced with a leucine zipper tetramerisation motif (Z) that allows Gag-Gag interactions for assembly, however, is devoid of RNA packaging.

Alternatively, as MOV10 is a putative RNA helicase, it may interact with HIV-1 viral RNA and affect cellular Gag expression in this manner. To investigate whether sequence-specific determinants and cis-acting elements within the HIV-1 genome are necessary for the reduction in virion production by MOV10 overexpression, a codon-optimised GPV-RRE construct was tested, in which the A-biased codon-usage of HIV-1 is swapped to allow optimal expression in mammalian cells.

4.3.2 NC, MA and sequence-specific elements of HIV-1 genomic RNA are dispensable for the decrease in VLP production by MOV10 overexpression

HeLa cells were co-transfected with either GPV-RRE, ΔNC+Z_t, ΔMA+M-src or codon-optimised GPV-RRE, together with pRev and pcDNA3.1-myc-MOV10 (pMOV10) or the pcDNA3.1-myc-GFP (pGFP) control. Cell lysates and sucrose-cushion purified VLPs were analysed by immunoblotting with anti-$p24^{Gag}$, anti-myc and anti-Hsp90 antibodies. As cell cultures were treated with the protease inhibitor saquinavir only a single $p55^{Gag}$ band was detected by immunoblotting. MOV10 overexpression consistently decreased the cellular Gag abundance and production of HIV-1 VLPs with the codon-optimised GPV-RRE (Figure 4.2B, lanes 3 and 4), ΔNC+Z_t (Figure 4.2B, lanes 5 and 6) and ΔMA+M-src (Figure 4.2B, lanes 7 and 8) constructs. Therefore, ectopically overexpressed MOV10 does not depend on NC or interfere with the RNA packaging function of NC for inhibition of VLP production. Similarly, the MA domain

of Gag and sequence-specific elements within the viral RNA are also dispensable for the mechanism by which MOV10 overexpression decreases HIV-1 Gag expression and VLP production.

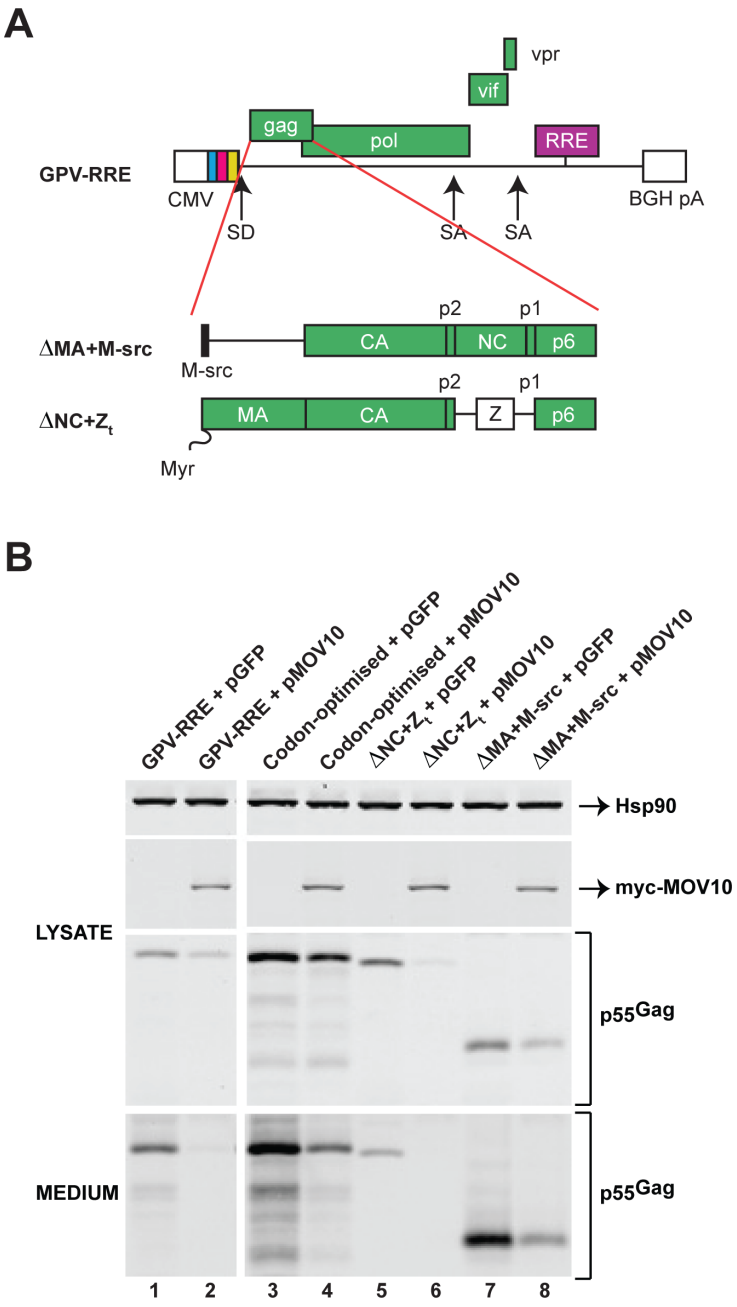


Figure 4.2. NC, MA and sequence-specific elements of HIV-1 genomic RNA are dispensable for the decrease in VLP production by MOV10 overexpression.

(A) GPV-RRE constructs. The GPV-RRE derived ΔMA+M-src construct lacks the MA domain, however, consists of a heterologous myristylation sequence derived from the *c-src* gene. The GPV-RRE based ΔNC+Z_t construct lacks the NC region and this is replaced with a leucine zipper tetramerisation motif (Z). **(B) NC, MA and sequence-specific elements of HIV-1 genomic RNA are dispensable for the decrease in VLP production by MOV10 overexpression.** 293T cells were co-transfected with either GPV-RRE, ΔMA+M-src, ΔNC+Z_t or a codon-optimised GPV-RRE construct (0.5 µg) together with pRev (0.25 µg) and pcDNA3.1-myc-MOV10 (pMOV10) or the pcDNA3.1-myc-GFP (pGFP) control (1.5 µg). Cell cultures were treated with the protease inhibitor saquinavir. Cell lysates were analysed by immunoblotting with anti-myc, anti-p24^{Gag} and anti-Hsp90 antibodies, and VLPs purified through a 20% sucrose cushion were also analysed by immunoblotting with an anti-p24^{Gag} antibody. Data are representative of 2 independent experiments.

4.4 MOV10 overexpression decreases HIV-1 genomic RNA packaging into virions

MOV10 is a putative RNA helicase, the cellular function of which is still unclear. Interestingly, MOV10 has been identified in several screens searching for interaction partners of various RBPs (Miki et al., 2011; Sim et al., 2012) and, moreover, a recent study has demonstrated the RNA-binding activity of MOV10 to be fairly broad and non-specific (Castello et al., 2012). Taking this into account, an interaction between MOV10 and HIV-1 viral RNA would not be surprising. As defective genome incorporation into nascent virions would compromise virion infectivity, the effect of MOV10 overexpression on HIV-1 viral RNA packaging was investigated as a potential mechanism by which ectopically overexpressed MOV10 may impact virion infectivity.

HeLa or 293T cells were co-transfected with pHIV-1_{NL4-3} and either pT7-MOV10 (pMOV10) or the pT7-GFP (pGFP) control. Equal amounts of virus normalised by the $p24^{Gag}$ concentration was sucrose-cushion purified, following which the RNA was extracted and cDNA was synthesised. The level of viral RNA packaged into virions was determined by quantitative polymerase chain reaction (qPCR) using a primer-probe set that recognises sequences in the HIV-1_{NL4-3} p6 domain. Ectopic overexpression of MOV10 decreased the level of HIV-1 genomic RNA detected in virions, which was reduced by approximately 40% and 60% for virions produced in HeLa and 293T cells, respectively, relative to the pGFP control (Figure 4.3). Harvested virion preparations and extracted RNA were treated with DNase to remove any residual transfected pHIV-1_{NL4-3} cDNA from the analysis. The efficiency of this treatment was verified by including duplicate samples minus the RT enzyme during the cDNA synthesis reaction and, subsequently, quantifying the level of viral cDNA in these samples by qPCR (Figure 4.3, denoted with an asterisk). Therefore, ectopically overexpressed MOV10 may interact with HIV-1 viral RNA directly or indirectly resulting in a decrease in HIV-1 genome packaging into virions that may wholly or partly contribute to the defect in virion infectivity.

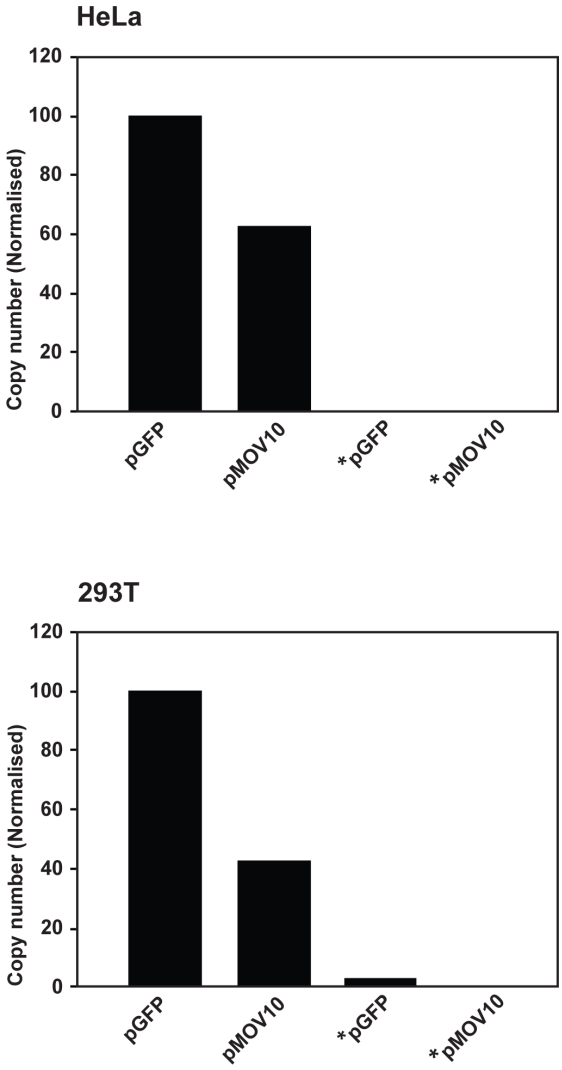


Figure 4.3. MOV10 overexpression decreases HIV-1 genomic RNA packaging into virions.

HeLa and 293T cells were co-transfected in 10cm tissue culture dishes with pHIV-1_{NL4-3} and pT7-MOV10 (pMOV10) or pT7-GFP (pGFP) at a DNA (μg) ratio of 3:1, respectively. Approximately 48hrs post-transfection virus-containing supernatant was harvested and treated with DNase and magnesium chloride for 2-3hrs. Virions were quantified by $p24^{Gag}$ ELISA as described in chapter 3 Figure 3.1B. Equal amounts of virus normalised by the $p24^{Gag}$ concentration was purified through a 20% sucrose cushion and the pelleted virions were lysed. RNA was extracted and treated with DNase for a further 30mins to digest any remaining input proviral plasmid DNA, and cDNA was synthesised from equal concentrations of the RNA by reverse-transcription polymerase chain reaction (RT-PCR). The viral RNA copy number in virions was measured by quantitative PCR (qPCR) using a primer-probe set that recognises sequences in the HIV-1_{NL4-3} p6 domain. Duplicate samples minus the RT enzyme were also included as controls to ensure that the majority of transfected proviral plasmid DNA was digested (*). Results are normalised to the pGFP control, which is set at 100%.

4.5 Ectopic overexpression of MOV10 produces HIV-1 virions defective in reverse transcription in target cells

The antiviral proteins A3F and A3G are packaged into assembling HIV-1 virions and impair reverse transcription in target cells by introducing C-to-U deamination events in the minus strand DNA that register as G-to-A hypermutations in the plus strand DNA, compromising the viral genome and HIV-1 infectivity (chapter 1 section 1.8.2.8). These proteins also inhibit the accumulation of viral cDNA by an editing-independent mechanism. Similarly, MOV10 is packaged into HIV-1 particles (Chertova et al., 2006), the incorporation of which has been reported to require an RNA-dependent interaction with the NC region of Gag (Burdick et al., 2010; Wang et al., 2010; Abudu et al., 2012). Considering the interaction of MOV10 with A3F or A3G, the effect of MOV10 overexpression on the efficiency of reverse transcription in target cells was determined.

Virus was produced by co-transfecting 293T cells with pHIV-1_{NL4-3} and either pT7-MOV10 (pMOV10) or the pT7-Luc (pLuc) control. Equal amounts of virus normalised by the $p24^{Gag}$ concentration was used to infect the HUT78 T cell line in a time-course, whereby cells were harvested at 0hrs, 8hrs, 24hrs and 48hrs post-infection. DNA was extracted from the cell lysates and the level of minus strand strong stop DNA was measured by qPCR using a primer-probe set that recognises sequences between R and the U5-PBS junction (Figure 4.4). MOV10 overexpression decreased the accumulation of minus strand strong stop DNA by approximately 20-fold at 8hrs and 24hrs, and by over 500-fold at 48hrs compared to the pLuc control (Figure 4.4). The significant increase in accumulation of reverse transcripts at 48hrs for the pLuc control is likely indicative of further rounds of spreading infection, which appear to be blocked with MOV10 overexpression (Figure 4.4). Furthermore, minus strand transfer and plus strand transfer DNA could not be detected with primer-probe sets recognising sequences in U3 and the U5-PBS junction or R and sequences just downstream of the PBS, respectively (data not shown). Therefore, MOV10 overexpression impairs the accumulation of HIV-1 reverse transcripts in target cells.

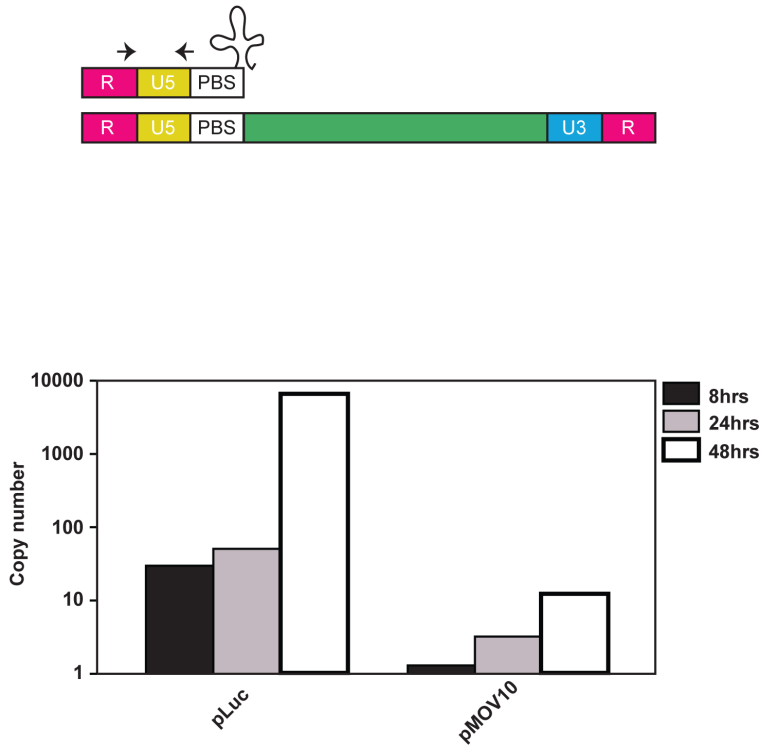


Figure 4.4. Ectopic overexpression of MOV10 produces HIV-1 virions defective in reverse transcription in target cells.

293T cells were co-transfected in 10cm tissue culture dishes with pHIV-1_{NL4-3} and pT7-MOV10 (pMOV10) or the pT7-Luc (pLuc) control at a DNA (µg) ratio of 3:1, respectively. Approximately 48hrs post-transfection virus was harvested and quantified by $p24^{Gag}$ ELISA as described in chapter 3 Figure 3.1B. Equal amounts of virus (20 ngs) normalised by the $p24^{Gag}$ concentration was used to spin-infect the HUT78 T cell line for 2hrs. Cells were harvested and lysed at 0hrs, 8hrs, 24hrs and 48hrs post spin-infection and DNA was extracted. Minus strand strong stop DNA copy numbers were measured by qPCR using a primer-probe set that recognises sequences between R and the U5-PBS junction (illustrated by black arrows in the top panel).

4.6 N-terminal and C-terminal MOV10 mutants

4.6.1 Overexpression of the N-terminal domain of MOV10 maintains the antiviral activity of full-length MOV10

MOV10 is a 1003 amino acid putative RNA helicase, the N-terminal 495 residues of which consist of no known protein motifs, and the C-terminal 508 amino acids of which contain the putative RNA helicase motifs (chapter 1 section 1.13). The results presented in this chapter so far demonstrate that ectopically overexpressed MOV10 inhibits the infectivity of HIV-1 virions by decreasing viral RNA packaging into virions and impairing reverse transcription, although additional as yet unidentified mechanisms may also contribute. These findings associate MOV10 with RNA stages of the HIV-1 life cycle, suggesting that the putative RNA helicase activity of MOV10 may be important for its antiretroviral function.

To test this, MOV10 N-terminal and C-terminal domain fragments were cloned into the pT7 plasmid generating pT7-MOV10-N (pMOV10-N) and pT7-MOV10-C (pMOV10-C). HeLa or 293T cells were co-transfected with pHIV-1_{NL4-3} and either full-length pT7-MOV10 (pMOV10), pMOV10-N, pMOV10-C or the pT7-Luc control (pLuc). The effect on virus production was quantified by $p24^{Gag}$ ELISA and results showed that overexpression of the N-terminal domain decreased HIV-1 virus production by over 80% for HeLa and 293T cells compared to the pLuc control (Figure 4.5). This percentage decrease in virion production was more potent than that observed with full-length MOV10 for HeLa cells, however, this is likely due to higher expression levels of the N-terminal protein relative to full-length MOV10 (Figure 4.5, lane 3). In contrast, the C-terminal protein is a loss of function mutant as overexpression of this domain did not decrease HIV-1 virus production, and even moderately enhanced it (Figure 4.5). To measure the effect on virion infectivity, equal amounts of virus normalised by the $p24^{Gag}$ concentration was added to TZM-bl cells. Similarly to the virus production results, overexpression of both full-length MOV10 and the N-terminal protein almost abolished HIV-1 infectivity, however, overexpression of the C-terminal protein had no effect on infectivity (Figure 4.5). Therefore, ectopic overexpression of the N-terminal domain of MOV10, and not the C-terminal putative helicase domain, maintains the anti-HIV-1 activity of full-length MOV10.

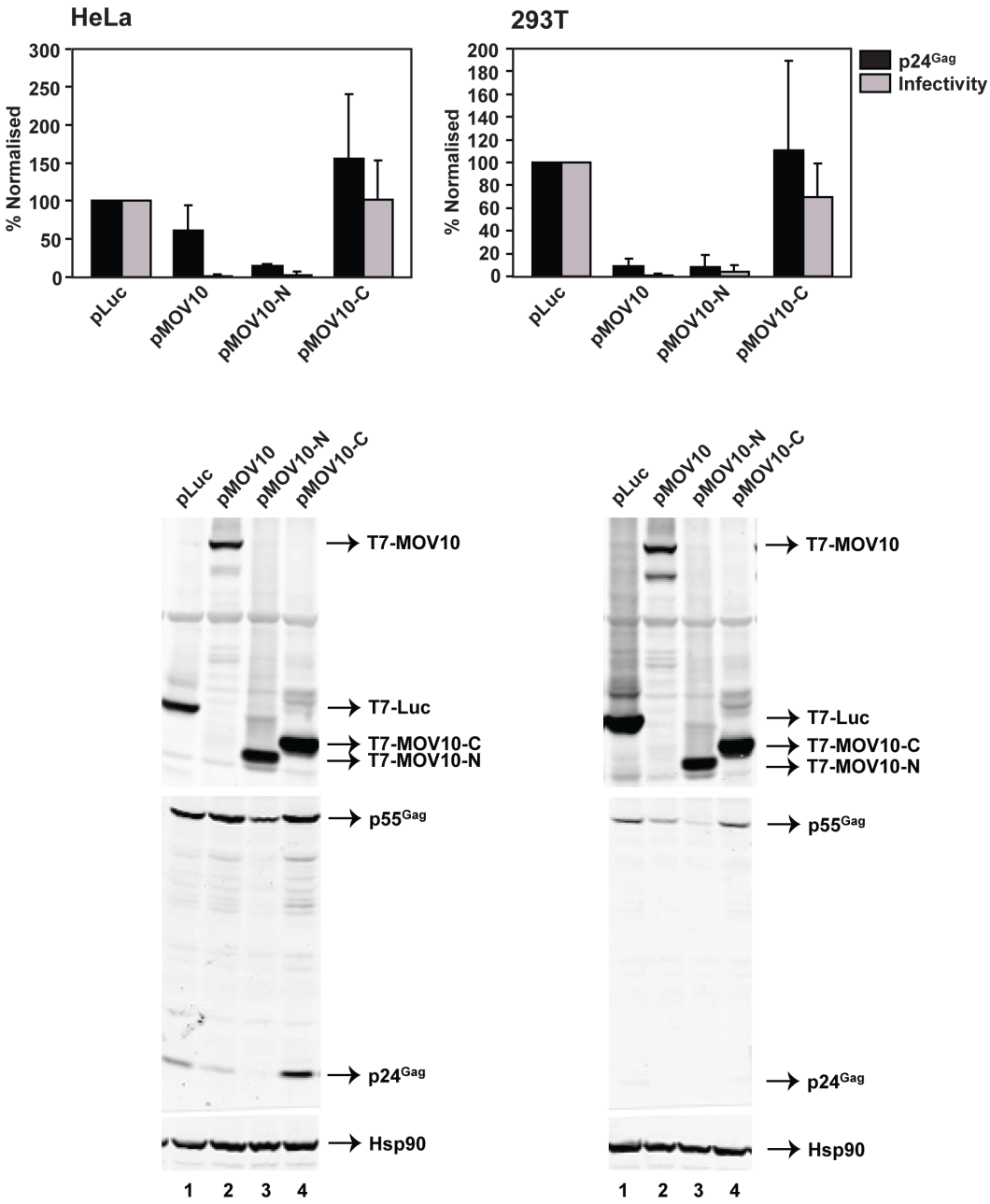


Figure 4.5. Overexpression of the N-terminal domain of MOV10 maintains the antiviral activity of full-length MOV10.

HeLa or 293T cells were co-transfected with pHIV-1_{NL4-3} (0.5 µg) and either full-length pT7-MOV10 (pMOV10), pT7-MOV10-N (pMOV10-N), pT7-MOV10-C (pMOV10-C) or the pT7-Luc control (pLuc) (1.5 µg). Virus production was quantified by $p24^{Gag}$ ELISA (black bars) and virion infectivity was measured by TZM-bl assay (grey bars) as described in chapter 3 Figure 3.1B. Cell lysates were analysed by immunoblotting with anti-T7, anti-$p24^{Gag}$ and anti-Hsp90 antibodies. Results are normalised to the pLuc control, which is set at 100%. Values are the mean ± SD of 4 independent experiments.

4.6.2 Ectopic overexpression of full-length MOV10 is essential for suppression of LINE-1 retrotransposition

MOV10 overexpression also potently suppresses the replication of endogenous retroelements (chapter 3 section 3.8); therefore, to determine the importance of MOV10 putative RNA helicase activity for this function, the effect of ectopically overexpressing the N-terminal and C-terminal MOV10 proteins on LINE-1 retrotransposition was investigated. HeLa cells were co-transfected with pJM101/L1.3 (pLINE-1) and either pT7-MOV10 (pMOV10), pMOV10-N, pMOV10-C or the pT7-Luc control (pLuc). Following G418 selection of cultures, enumeration of cell colonies revealed over an 80% and 40% decrease in LINE-1 retrotransposition with overexpression of the N-terminal and C-terminal proteins, respectively, relative to the pLuc control (Figure 4.6). On the other hand, overexpression of full-length MOV10 almost completely suppressed LINE-1 retrotransposition (Figure 4.6). These results indicate that overexpression of both the N-terminal and C-terminal domains of MOV10 is necessary for efficient inhibition of LINE-1 propagation.

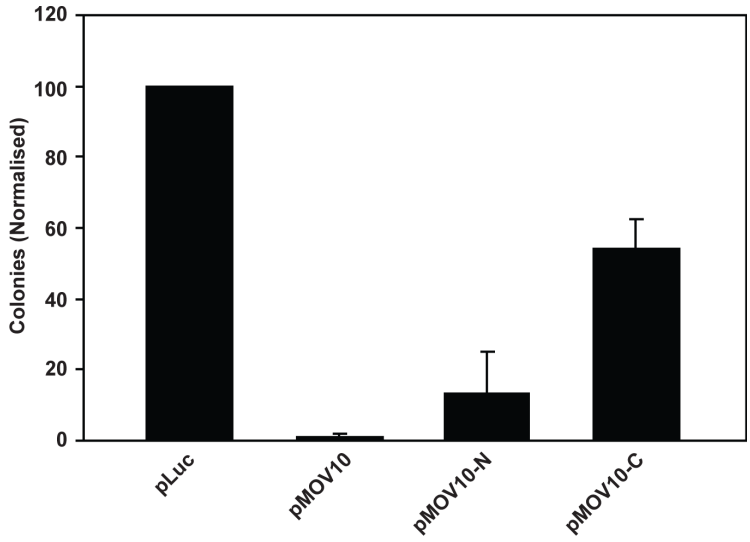


Figure 4.6. Ectopic overexpression of full-length MOV10 is essential for suppression of LINE-1 retrotransposition.

HeLa cells were co-transfected with pJM101/L1.3 (pLINE-1) (1.5 μg) containing the *neo* cassette as described in chapter 3 Figure 3.9, and either full-length pT7-MOV10 (pMOV10), pT7-MOV10-N (pMOV10-N), pT7-MOV10-C (pMOV10-C) or the pT7-Luc control (pLuc) (1 μg). The retrotransposition frequency was determined by G418 selection of the cultures as described in chapter 3 Figure 3.9. Results are normalised to the pLuc control, which is set at 100%. Values are the mean ± SD of 3 independent experiments.

4.6.3 Subcellular localisation of N-terminal and C-terminal MOV10 proteins varies from that of full-length MOV10

The MOV10 N-terminal and C-terminal proteins consist of sizeable deletions that may have affected the subcellular distribution and, therefore, function of these mutants in comparison with full-length MOV10. Therefore, to assess the localisation of MOV10 and MOV10 mutants, HeLa cells were transfected with pT7-MOV10 (pMOV10), pMOV10-N or pMOV10-C following which cells were fixed and analysed by immunofluorescence using an anti-T7 antibody. Cells were also stained with an antibody against the P body marker DDX6. The distribution of full-length MOV10 was cytoplasmically diffuse and punctate with MOV10 strongly co-localising with DDX6 in P bodies (Figure 4.7A). In contrast, the N-terminal protein did not localise to P bodies and, moreover, disrupted P bodies as well (Figure 4.7B, top panel). This differential localisation may be attributed to higher expression levels of the N-terminal protein in comparison with full-length MOV10 as determined by immunoblotting (Figure 4.5). Similarly, the C-terminal protein did not localise to P bodies either, although this mutant had no effect on P bodies unlike that observed for the N-terminal protein (Figure 4.7B, lower panel). Additionally, the C-terminal protein also localised to the nucleus. These results demonstrate that the expression levels and subcellular localisation of truncated N-terminal and C-terminal MOV10 proteins varies greatly from that of full-length MOV10, questioning the reliability of these mutant proteins in assessing wild-type MOV10 function.

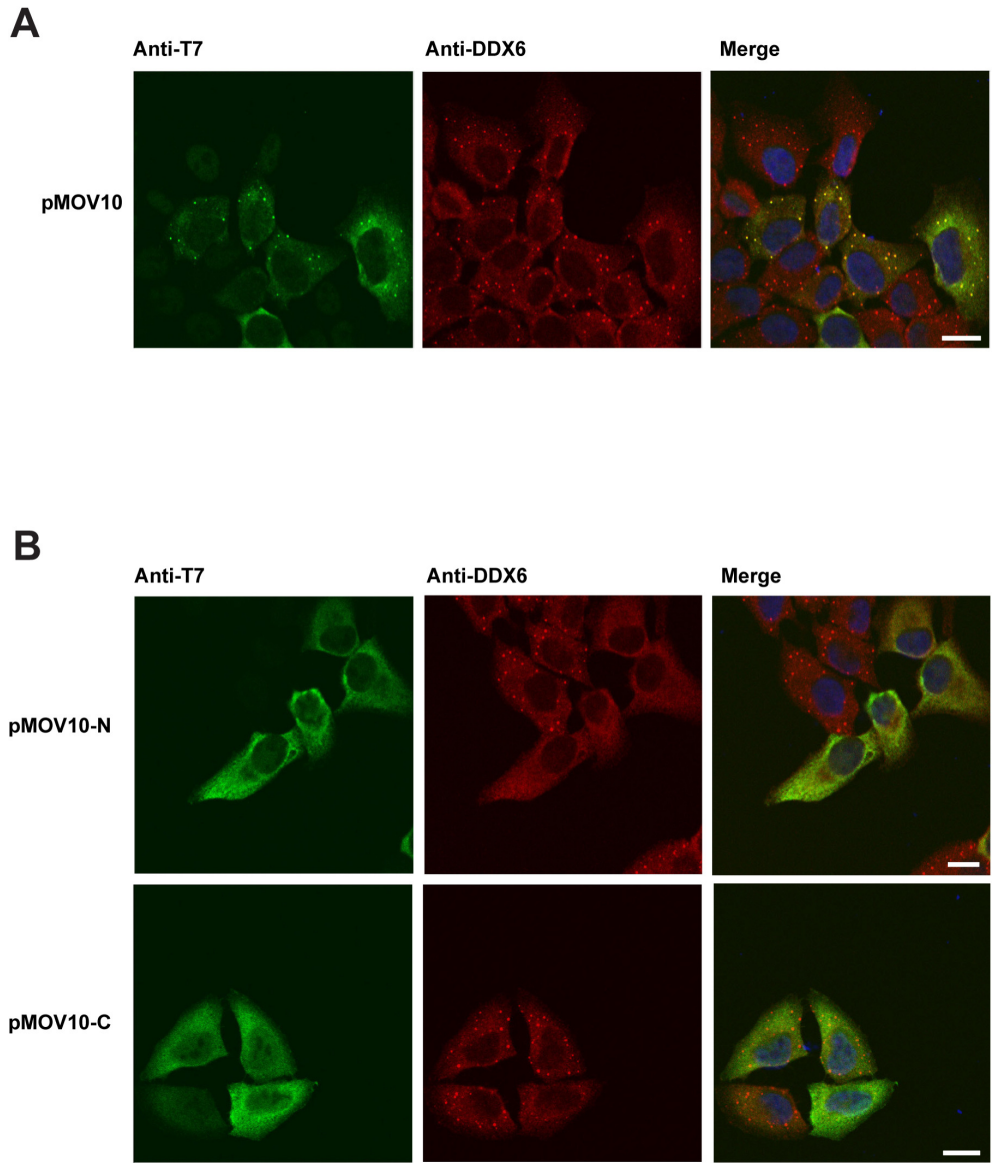


Figure 4.7. Subcellular localisation of N-terminal and C-terminal MOV10 proteins varies from that of full-length MOV10.

(A) Full-length MOV10 is cytoplasmically diffuse and in P bodies. HeLa cells were plated onto coverslips and co-transfected with full-length pT7-MOV10 (pMOV10) (1.5 µg). Approximately 24hrs post-transfection cells were fixed in 4% PFA and stained with an anti-T7 primary antibody and appropriate Alexa Fluor conjugated secondary antibody, as well as DAPI. Coverslips were mounted onto slides and analysed by immunofluorescence. **(B) Subcellular distribution of N-terminal and C-terminal MOV10 mutants varies from that of full-length MOV10.** HeLa cells were plated onto coverslips and co-transfected with pT7-MOV10-N (pMOV10-N) or pT7-MOV10-C (pMOV10-C) (1.5 µg). Coverslips were analysed by immunofluorescence as described in panel (A). Data are representative of 2 independent experiments. Scale bar = 10µm.

4.7 Full-length MOV10 putative helicase mutants

4.7.1 MOV10 putative helicase mutants

Judging from the experiments with N-terminal and C-terminal MOV10 proteins the putative RNA helicase activity of MOV10 appears to be dispensable for MOV10 antiviral function and, furthermore, both the N-terminal and C-terminal proteins seem to be required for suppression of LINE-1 retrotransposition to levels observed with full-length MOV10. However, the expression levels and subcellular localisation of these truncated MOV10 mutants varied greatly from that of full-length MOV10 making the interpretation of these data somewhat difficult. Consequently, full-length MOV10 putative helicase mutants were generated to assess the contribution of MOV10 putative RNA helicase activity for the inhibition of HIV-1 and LINE-1 replication. Fairman-Williams et al aligned SF1 helicases from *Escherichia coli*, yeast and humans [reviewed in (Fairman-Williams et al., 2010)], and analysis of these alignments revealed highly conserved residues in the Walker A and Walker B putative helicase motifs of MOV10. Subsequently three MOV10 Walker A and Walker B putative helicase mutants were generated by alanine substitution of (1) the conserved lysine residue at position 530 (K530A), (2) the conserved aspartic acid and glutamic acid residues at positions 645 and 646 (DE645AA) and (3) the less well conserved glycine amino acid at position 648 (G648A) (Figure 4.8). Upf1 is a SF1 helicase, and the latter mutation was based on the published Upf1 Walker B ATPase mutant (DESTQ to DEAAQ) (Franks et al., 2010). The putative helicase mutants were cloned into the pT7 plasmid generating pT7-MOV10-K530A (pMOV10-K530A), pT7-MOV10-DE645AA (pMOV10-DE645AA) and pT7-MOV10-G648A (pMOV10-G648A).

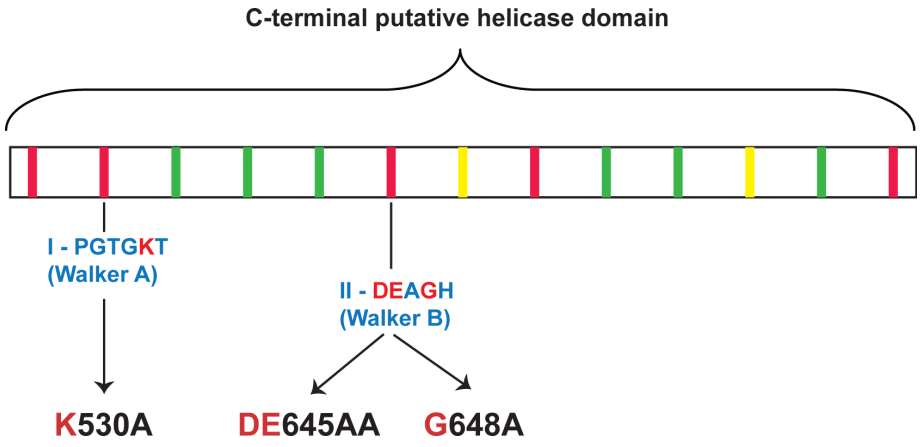


Figure 4.8. MOV10 putative helicase mutants.

Three MOV10 Walker A and Walker B putative helicase mutants were generated. K530A is a Walker A mutant in which the lysine residue (K) is substituted with an alanine (A). DE645AA is a Walker B mutant in which the aspartic acid and glutamic acid residues (DE) are replaced with alanines (AA). G648A is also a Walker B mutant in which the glycine residue (G) is substituted with an alanine (A).

4.7.2 Overexpression of K530A and DE645AA only partially maintains the antiviral activity of wild-type MOV10

To assess whether MOV10 depends on its putative RNA helicase activity for inhibition of HIV-1, 293T cells were co-transfected with pHIV-1_{NL4-3} and either pT7-MOV10 (pMOV10), pMOV10-K530A, pMOV10-DE645AA, pMOV10-G648A or the pT7-Luc (pLuc) control. The virion concentration was determined by $p24^{Gag}$ ELISA and results showed an approximately 60% and 40% decrease in virion production with K530A and DE645AA overexpression, respectively, relative to the pLuc control (Figure 4.9). In comparison, wild-type MOV10 overexpression decreased virion production by approximately 90% demonstrating that the K530A and DE645AA proteins are partial loss of function mutants (Figure 4.9). In contrast, overexpression of the G648A mutant maintained the antiviral activity of wild-type MOV10 (Figure 4.9). Variations in expression level did not account for these differential observations as all three MOV10 mutants were expressed at similar levels as wild-type MOV10 (Figure 4.9, compare lane 2 with 3, 4 and 5). The effect of overexpressing these MOV10 putative helicase mutants on the infectivity of HIV-1 virions was determined by TZM-bl assay. Similar to the observations for virion production, ectopic overexpression of wild-type MOV10 and G648A almost abolished infectivity completely, whereas overexpression of K530A and DE645AA reduced infectivity by approximately 80% and 40%, respectively, compared to the pLuc control (Figure 4.9). Therefore, K530A and DE645AA are partial loss of function MOV10 mutants implying that MOV10 putative RNA helicase activity may be necessary for its antiviral function.

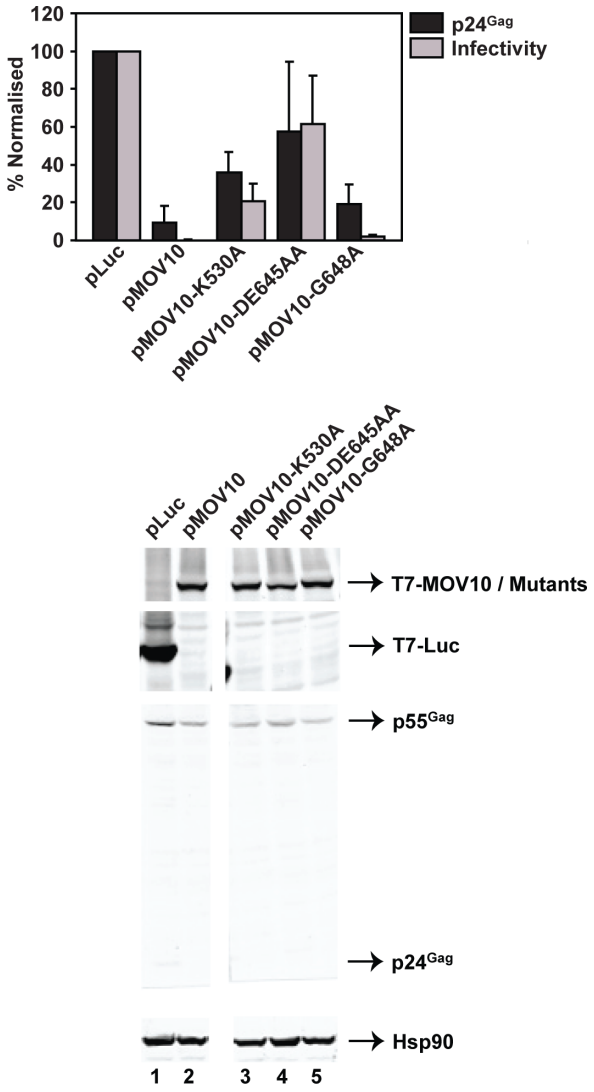


Figure 4.9. Overexpression of K530A and DE645AA only partially maintains the antiviral activity of wild-type MOV10.

293T cells were co-transfected with pHIV-1_{NL4-3} (0.5 µg) and either wild-type pT7-MOV10 (pMOV10), pT7-MOV10-K530A (pMOV10-K530A), pT7-MOV10-DE645AA (pMOV10-DE645AA), pT7-MOV10-G648A (pMOV10-G648A) or the pT7-Luc control (pLuc) (1.5 µg). Virus production was quantified by $p24^{Gag}$ ELISA (black bars) and virion infectivity was measured by TZM-bl assay (grey bars) as described in chapter 3 Figure 3.1B. Cell lysates were analysed by immunoblotting with anti-T7, anti-$p24^{Gag}$ and anti-Hsp90 antibodies. Results are normalised to the pLuc control, which is set at 100%. Values are the mean ± SD of 3 independent experiments.

4.7.3 Overexpression of K530A and DE645AA only partially maintains the capacity of wild-type MOV10 to suppress LINE-1 retrotransposition

Similarly, the contribution of MOV10 putative RNA helicase activity for MOV10-mediated suppression of endogenous retroelements was determined by testing the effect of ectopically overexpressing these MOV10 mutants on LINE-1 retrotransposition. HeLa cells were co-transfected with pJM101/L1.3 (pLINE-1) and either pT7-MOV10 (pMOV10), pMOV10-K530A, pMOV10-DE645AA, pMOV10-G648A or the pT7-Luc control (pLuc). Cell cultures were G418 selected and the colonies were counted and, similar to the observations for HIV-1, overexpression of wild-type MOV10 and G648A inhibited LINE-1 retrotransposition completely, however, ectopic overexpression of K530A and DE645AA reduced retrotransposition by approximately 60% and 30% respectively, compared to the pLuc control (Figure 4.10). These results demonstrate that highly conserved residues within the MOV10 Walker A and Walker B putative RNA helicase motifs are necessary for efficient MOV10-mediated inhibition of both HIV-1 and LINE-1 replication, which implies that MOV10 putative RNA helicase activity may be important for MOV10 anti-retroelement activity.

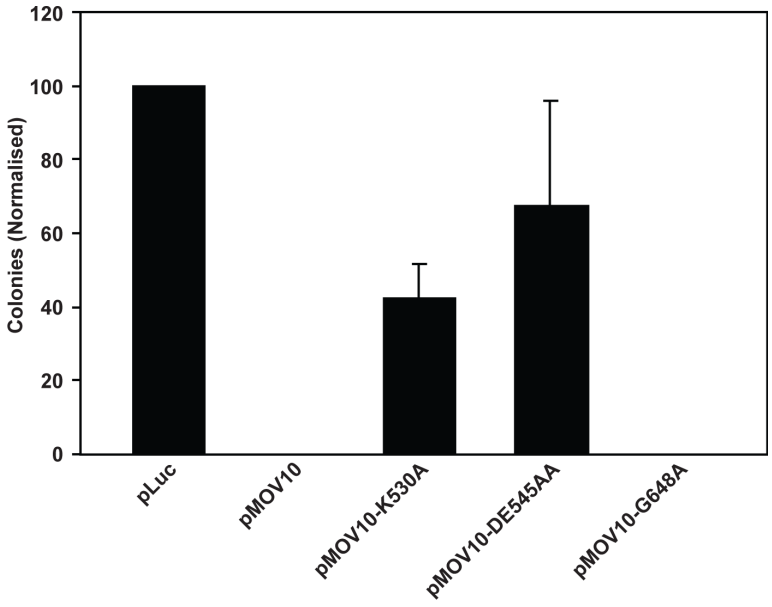


Figure 4.10. Overexpression of K530A and DE645AA only partially maintains the capacity of wild-type MOV10 to suppress LINE-1 retrotransposition.

HeLa cells were co-transfected with pJM101/L1.3 (pLINE-1) (1.5 μg) consisting of the *neo* cassette as described in chapter 3 Figure 3.9, and either wild-type pT7-MOV10 (pMOV10), pT7-MOV10-K530A (pMOV10-K530A), pT7-MOV10-DE645AA (pMOV10-DE645AA), pT7-MOV10-G648A (pMOV10-G648A) or the pT7-Luc control (pLuc) (1 μg). The retrotransposition frequency was determined by G418 selection of the cultures as described in chapter 3 Figure 3.9. Results are normalised to the pLuc control, which is set at 100%. Values are the mean ± SD of 3 independent experiments.

4.7.4 Subcellular localisation of MOV10 putative helicase mutants

The subcellular distribution of the MOV10 putative helicase mutants was determined by transfecting HeLa cells with pMOV10-K530A, pMOV10-DE645AA or pMOV10-G648A, and cells were fixed and analysed by immunofluorescence using an anti-T7 antibody. Again, cells were also stained with an anti-DDX6 antibody, which serves as a marker for P bodies. Interestingly, the K530A and DE645AA mutants showed a similar distribution to that observed for the N-terminal MOV10 protein, whereby both mutants were cytoplasmically diffuse and also disrupted P bodies (Figure 4.11, top and middle panel). K530A and DE645AA mutants were expressed at similar levels to wild-type MOV10 as determined by immunoblotting (Figure 4.9), indicating that the varied distribution observed for the N-terminal MOV10 protein was unlikely due to its higher expression levels (Figure 4.7B). In contrast, the subcellular localisation of the functional G648A mutant reflected that of wild-type MOV10, whereby G648A was cytoplasmically diffuse and co-localised with DDX6 in P bodies (Figure 4.11, lower panel and Figure 4.7A). Therefore, substitution of just one or two conserved residues within the MOV10 Walker A or Walker B motif is sufficient to alter the subcellular distribution of MOV10 and impact on its anti-retroelement activity, implying that the localisation of MOV10 may be important for its function.

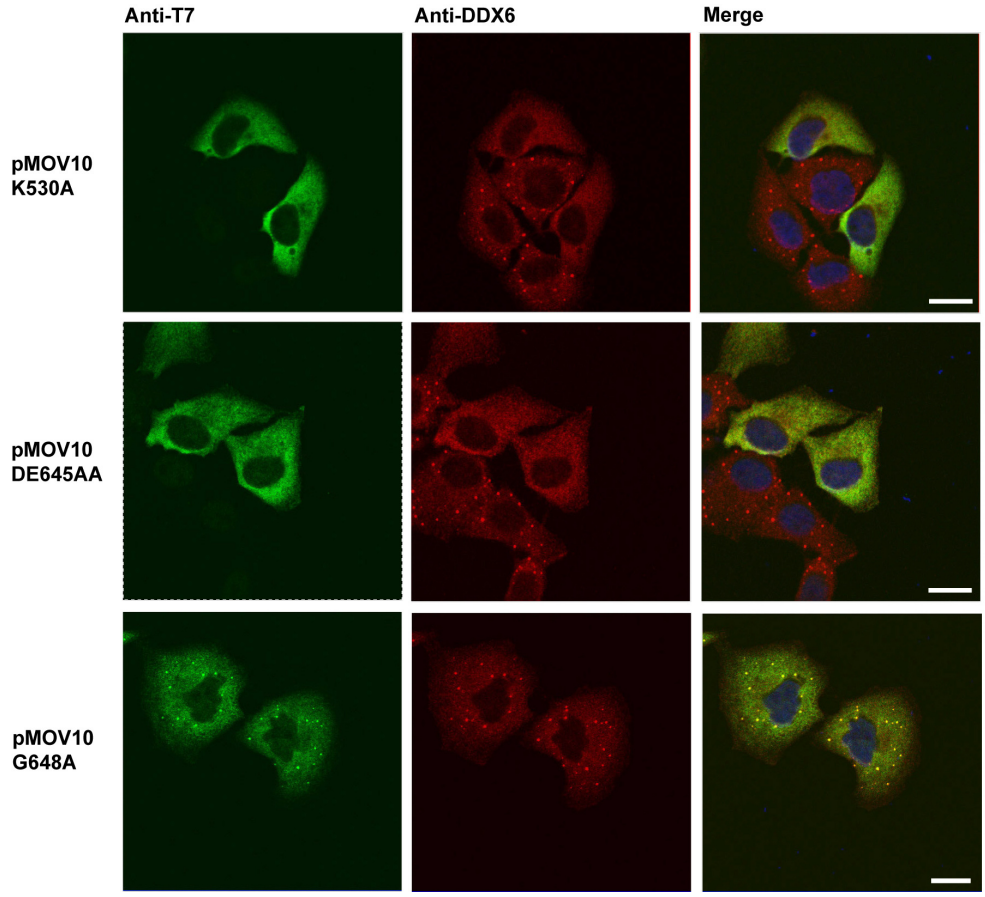


Figure 4.11. Subcellular localisation of MOV10 putative helicase mutants.

HeLa cells were plated onto coverslips and co-transfected with either pT7-MOV10-K530A (pMOV10-K530A), pT7-MOV10-DE645AA (pMOV10-DE645AA) or pT7-MOV10-G648A (pMOV10-G648A) (1.5 µg). Wild-type MOV10 subcellular localisation is described in (Figure 4.7A). Approximately 24hrs post-transfection cells were fixed in 4% PFA and stained with an anti-T7 primary antibody and appropriate Alexa Fluor conjugated secondary antibody, as well as DAPI. Coverslips were mounted onto slides and analysed by immunofluorescence. Scale bar = 10µm.

4.8 Discussion

The capacity of MOV10 overexpression to restrict the replication of a broad panel of genetically diverse exogenous and endogenous retroelements ranging from HIV-1 to LINE-1 provides clues for its mode of action, such that MOV10 may target a factor or step common in the life cycle of these retroviruses and retrotransposons. This chapter aimed to identify some of these targets and, secondly, focused on exploring the importance of MOV10 putative RNA helicase activity for its anti-retroelement function through structure-function analyses.

MOV10 overexpression decreases the cellular Gag abundance of HIV-1, HIV-2 and M-PMV (chapter 3 sections 3.4, 3.6 and 3.7) and this may contribute to the decrease in virus production. Immunofluorescence studies revealed that ectopically overexpressed MOV10 and HIV-1 Gag co-localise in cytoplasmic aggregates that resemble SGs (Figure 4.1). Although further studies with appropriate SG markers and controls will be necessary to confirm whether MOV10 and Gag indeed co-localise in SGs, this finding may be suggestive of a potential mechanism by which ectopically overexpressed MOV10 may decrease cellular Gag abundance through an association with Gag, or even sequestration of it, in cytoplasmic aggregations. Interestingly, MOV10 was recently identified in a screen as a HIV-1 Gag interaction partner, although the nature of this interaction was not investigated further (Engeland et al., 2011). In the event that an association between MOV10 and Gag is responsible for the decrease in HIV-1 virion production, the results presented in this chapter reveal that the NC and MA domains of Gag are dispensable for such an interaction as ectopically overexpressed MOV10 effectively decreases the cellular Gag expression and production of HIV-1 VLPs lacking these domains (Figure 4.2B). Furthermore, the NC domain is responsible for packaging of the viral RNA into virions, which confirms that MOV10 overexpression does not decrease HIV-1 VLP production by interfering with RNA packaging either (Figure 4.2B).

As an alternative hypothesis, considering that MOV10 is a putative RNA helicase that has also been reported to function in miRNA-mediated post-transcriptional RNA silencing pathways (Meister et al., 2005), ectopically overexpressed MOV10 may interact with unspliced viral transcripts and inhibit Gag translation directly. However, this potential interaction between MOV10 and viral RNA must be independent of the

viral RNA sequence and cis-acting elements as MOV10 overexpression effectively decreases the production of HIV-1 VLPs produced from a codon-optimised construct as well (Figure 4.2B). This is not unlikely considering the broad RNA binding ability of MOV10 (Castello et al., 2012).

Ectopic overexpression of MOV10 not only decreases the production of HIV-1 virions, but also potently reduces the infectivity of virions produced (chapter 3 section 3.4), and this is also true for HIV-2 (chapter 3 section 3.6). MOV10 overexpression inhibits the production of infectious SIVmac, M-PMV and MLV particles as well, therefore, this finding may extend to these retroviruses; however, this could not be confirmed for all retroviruses tested, as the respective assays used did not differentiate between an effect on virus production or infectivity. MOV10 overexpression decreases the level of HIV-1 genomic RNA packaged into virions (Figure 4.3), and whether this affects viral RNA dimerisation remains to be established. The association of MOV10 with RNA metabolism pathways may account for this effect although further studies will be required to verify this. Compared to the control, HIV-1 genome incorporation is reduced by approximately 40% and 60% in virions produced from HeLa and 293T cells, respectively (Figure 4.3), however, at a similar concentration of pMOV10, infectivity of HIV-1 virions is reduced more potently. These results suggest that either the effect on viral RNA packaging is amplified in terms of its impact on virus infectivity, or alternatively, ectopically overexpressed MOV10 inhibits the infectivity of HIV-1 virions by more than one mechanism.

Proteomics studies detected endogenous MOV10 in HIV-1 virions produced from infected monocyte-derived macrophages (MDMs) (Chertova et al., 2006). More recently studies have identified this interaction to be dependent on the NC domain of Gag and residues within the N-terminal domain of MOV10, as well as putative helicase motifs in the MOV10 C-terminal domain (Wang et al., 2010; Abudu et al., 2012). Furthermore, MOV10 interacts with members of the APOBEC3 family that restrict HIV-1 infection by interfering with the process of reverse transcription. Consistently, MOV10 overexpression produces virions that are defective in the accumulation of minus strand strong stop DNA (Figure 4.4) and also late reverse transcription products in target cells (data not shown). Other groups have reported similar decreases in the synthesis of early and late reverse transcripts (Burdick et al., 2010; Furtak et al., 2010;

Wang et al., 2010), however, it is not yet clear whether this is a consequence of reduced genome packaging, or whether MOV10 may directly impede reverse transcription. Interestingly, ectopically overexpressed MOV10 also decreases the accumulation of IAP reverse transcription products and inhibits IAP retrotransposition, although has no effect on IAP RNA packaging into VLPs (Lu et al., 2012). Therefore, reverse transcription may be a common target for MOV10-mediated restriction of diverse exogenous and endogenous retroelements.

These results associate MOV10 with RNA stages of the HIV-1 life cycle and, therefore, MOV10 putative RNA helicase activity may be important for this function. Early structure-function studies revealed that overexpression of the N-terminal domain of MOV10, which contains no known protein motifs, decreases the production and infectivity of HIV-1 virions as effectively as full-length MOV10 (Figure 4.5). In sharp contrast, overexpression of the C-terminal putative RNA helicase domain of MOV10 has no effect on HIV-1 virus production or infectivity, and in fact enhances virus production slightly (Figure 4.5). These results suggest that the antiviral activity of MOV10 is localised to its N-terminal domain. Furtak et al reported similar findings with the overexpression of N-terminal and C-terminal MOV10 proteins (Furtak et al., 2010). However, the reliability of these results is questionable as both the N-terminal and C-terminal proteins are expressed at higher levels relative to full-length MOV10 making any direct extrapolations regarding MOV10 inhibitory function inaccurate (Figure 4.5). Moreover, unlike full-length MOV10 the C-terminal protein also localises to the nucleus (Figure 4.7B, lower panel) suggesting that such large truncations can drastically alter the subcellular localisation of proteins and, therefore, their function as well. In the case of LINE-1 replication, both the N-terminal and C-terminal proteins suppress LINE-1 retrotransposition, however, not as effectively as the full-length protein (Figure 4.6), suggesting that both domains may be important for restriction of LINE-1 mobilisation. Alternatively, mislocalisation of the C-terminal protein to the nucleus may interfere with steps such as TPRT, producing a phenotype unrepresentative of full-length MOV10 activity.

The MOV10 putative helicase mutants reflect a better system in comparison with the N-terminal and C-terminal proteins for such structure-function analyses, and these proteins are expressed at levels similar to wild-type MOV10 as well (Figure 4.9). Interestingly,

the K530A and DE645AA mutants are partial loss of function MOV10 mutants as overexpression of these proteins decreased HIV-1 virus production and infectivity less effectively than wild-type MOV10 (Figure 4.9). In contrast, G648A is a functional MOV10 mutant with antiviral activity comparable to wild-type MOV10 (Figure 4.9). These results suggest that the conserved lysine residue in the Walker A motif and the aspartic acid and glutamic acid residues in the Walker B motif are necessary for MOV10-mediated inhibition of HIV-1 virus production and infectivity. Similarly, these residues are also critical for the suppression of LINE-1 retrotransposition as ectopically overexpressed K530A and DE645AA mutants suppress LINE-1 retrotransposition less effectively than wild-type MOV10 (Figure 4.10).

Intriguingly, Furtak et al substituted just the conserved glutamic acid residue in the Walker B motif with a glutamine (E646Q), and showed that ectopically overexpressed E646Q decreased the infectivity of HIV-1 virions akin to wild-type MOV10 (Furtak et al., 2010). Therefore, it may be that the aspartic acid residue in the Walker B motif alone is essential for MOV10 antiviral activity. Although the use of different substitutions i.e. glutamine instead of alanine complicates the direct comparison of these data. Furthermore, as K530A and DE645AA are only partial loss of function MOV10 mutants this implies that residues within other putative helicase motifs or even the N-terminal domain of MOV10 may contribute to anti-retroelement activity. Consistently, Wang et al tested several mutants generated by substituting alanines in place of key residues within seven putative helicase motifs, and reported that the ectopic overexpression of each mutant decreased HIV-1 infectivity less effectively than wild-type MOV10 (Wang et al., 2010). Furthermore, Abudu et al mapped the antiviral region of MOV10 to amino acids 99-949 indicating that residues spanning the majority of MOV10 may be necessary for its inhibitory activity (Abudu et al., 2012).

Interestingly, the subcellular distribution of K530A, DE645AA and also the N-terminal MOV10 protein varies from that of wild-type MOV10, whereby these proteins do not localise to P bodies and, furthermore, appear to either prevent the formation of P bodies or disrupt them (Figures 4.7 and 4.11). In contrast, the subcellular localisation of G648A reflects that of wild-type MOV10. These results suggest that these conserved residues within the Walker A and Walker B motifs may be important for P body formation and/or stability. Furthermore, the N-terminal domain of MOV10 may

function in mediating the localisation of MOV10 to P bodies, as the C-terminal protein alone does not localise to DDX6 P bodies (Figure 4.7B, lower panel), although further studies will be necessary to confirm this. Secondly, as K530A and DE645AA are partial loss of function MOV10 mutants, it is tempting to speculate that P bodies or P body factors may be important for the anti-retroelement activity of MOV10, although Lu et al recently reported MOV10 overexpression to effectively suppress IAP replication in the absence of P bodies as well (Lu et al., 2012).

CHAPTER 5
RESULTS

Endogenous MOV10 suppresses endogenous retroelements, but not exogenous retroviruses

5.1 Introduction

Although MOV10 overexpression potently restricts retroviruses and retrotransposons, these data do not confirm a physiological role for endogenous MOV10 in the control of retroelements, and are simply indicative of potential MOV10 anti-retroelement activity. Therefore, the effect of silencing endogenous MOV10 by RNAi on the replication of exogenous and endogenous retroelements was investigated. Additionally, the Type I IFN response is activated during viral infections and may also be triggered in response to intracellular genetic parasites in the absence of necessary host regulatory factors [reviewed in (Pitha, 2011)](Stetson et al., 2008). This family of cytokines blocks both early and late stages of viral replication through the onset of an antiviral state mediated by the upregulation of antiviral IFN-stimulatory genes (ISGs) as well as the activation of myeloid and lymphoid cells important for combating infection [reviewed in (Stetson and Medzhitov, 2006; Pitha, 2011)](Goujon and Malim, 2010). Members of the APOBEC3 family of proteins and tetherin are induced by IFNα treatment (Neil et al., 2007; Koning et al., 2009); therefore, the effect of IFNα treatment on MOV10 protein expression was assessed to address whether MOV10 may also be stimulated by IFNα to levels sufficient for the natural restriction of retroelements. The requirement of MOV10 for miRNA or siRNA-mediated post-transcriptional RNA silencing pathways, formation of cytoplasmic P bodies and APOBEC3 protein function (Meister et al., 2005; Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008) was also investigated through MOV10 knockdown studies as a way to better understand the cellular function of MOV10, and further delve into the mechanism of MOV10's anti-retroelement activity.

5.2 Effect of depleting endogenous MOV10 on HIV-1 replication

5.2.1 Silencing endogenous MOV10 has no effect on HIV-1 virus production or the infectivity of virions produced

MOV10 knockdown (KD) cell lines were produced by transducing HeLa or 293T cells with lentiviral vectors expressing either a non-silencing control short hairpin RNA (shRNA) or a MOV10-specific shRNA. These vectors also encoded a puromycin resistance gene and, therefore, successfully transduced cells were selected with puromycin treatment. HeLa or 293T non-silencing control and MOV10 KD cell lysates were analysed by immunoblotting with an anti-MOV10 antibody to determine the efficiency of MOV10 KD, and MOV10 could not be detected at the protein level even when the amount of lysate analysed was greatly increased (Figure 5.1A). Furthermore, the growth rate of MOV10 KD cells was similar to that of the non-silencing control cells (data not shown). MOV10 overexpression decreases the production of HIV-1 virions and also inhibits the infectivity of virions produced. To assess whether natural levels of MOV10 similarly restrict HIV-1 the effect of depleting endogenous MOV10 on the production and infectivity of HIV-1 virions was investigated. For this HeLa or 293T non-silencing control and MOV10 KD cells were transfected with pHIV-1_{NL4-3} and virion production and infectivity were determined by $p24^{Gag}$ ELISA and TZM-bl assay, respectively. Silencing of endogenous MOV10 had no effect on the production (Figure 5.1A) or infectivity (Figure 5.1B) of HIV-1 virions suggesting that endogenous levels of MOV10 do not control HIV-1 virus replication.

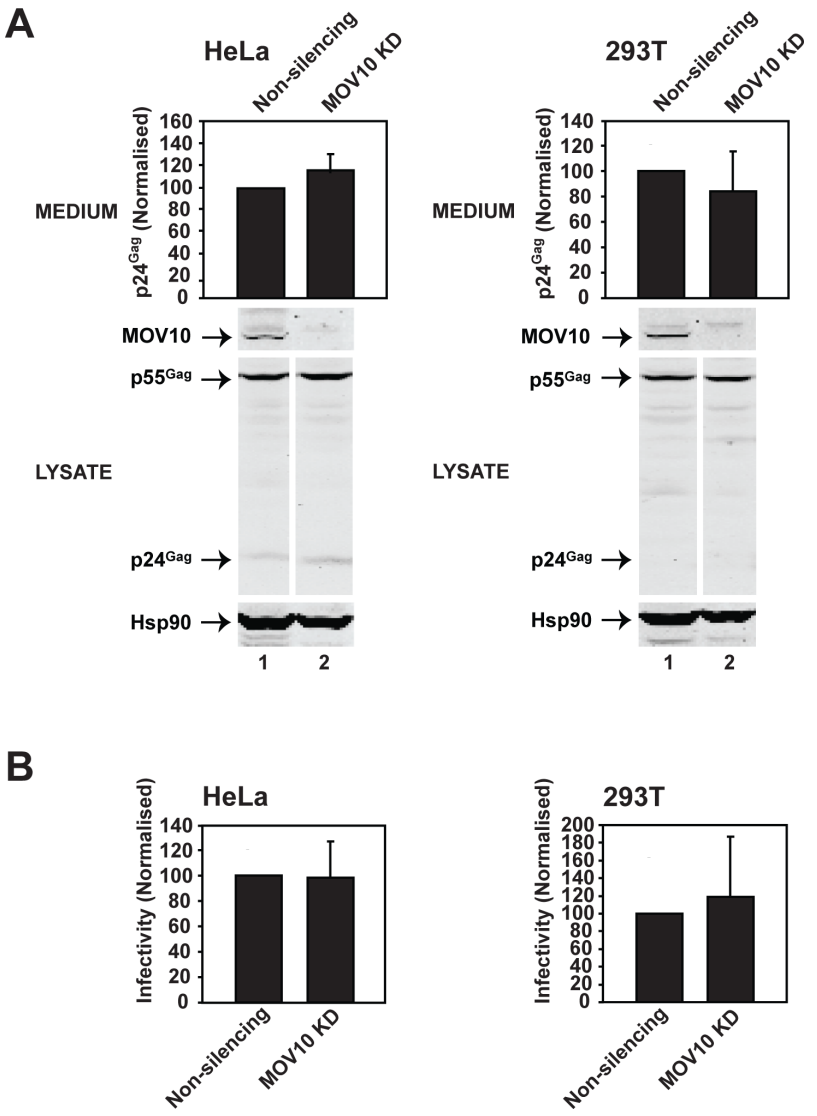


Figure 5.1. Silencing endogenous MOV10 has no effect on HIV-1 virus production or the infectivity of virions produced.

(A) Silencing endogenous MOV10 has no effect on HIV-1 virus production. MOV10 knockdown (KD) cell lines were produced by transducing HeLa or 293T cells with lentiviral vectors expressing either a non-silencing control shRNA or a MOV10-specific shRNA. Cell cultures were treated with puromycin to select for successfully transduced cells. HeLa or 293T non-silencing control or MOV10 KD cells were transfected with pHIV-1_{NL4-3} (1 μg). Virus production was quantified by $p24^{Gag}$ ELISA as described in chapter 3 Figure 3.1B. Cell lysates were analysed by immunoblotting with anti-MOV10, anti-$p24^{Gag}$ and anti-Hsp90 antibodies. **(B) Endogenous MOV10 KD has no effect on the infectivity of HIV-1 virions produced**. The infectivity of equal amounts of virus normalised by the $p24^{Gag}$ concentration from the experiments in panel (A) was measured by TZM-bl assay as described in chapter 3 Figure 3.1B. Results are normalised to the non-silencing control, which is set at 100%. Values are the mean ± SD of 4 independent experiments.

5.2.2 Depletion of endogenous MOV10 has no effect on HIV-1 virus production or the infectivity of virions produced (infection assay)

Production of HIV-1 virions through the transfection of cells with pHIV-1_{NL4-3} does not reflect physiological infection, and may saturate any potential subtle phenotypes attributed to the depletion of endogenous MOV10. For this reason the effect of endogenous MOV10 depletion on HIV-1 virus production and infectivity was established with an alternative assay whereby producer cells were infected with virus as opposed to transfected with pHIV-1_{NL4-3}, resembling natural infection more closely. For the infection assay, VSV-G pseudotyped HIV-1 virions were produced in 293T cells by co-transfecting cells with pHIV-1_{NL4-3} and pVSV-G (Figure 5.2). Equal amounts of virus normalised by the $p24^{Gag}$ concentration (MOI 0.3) was used to infect HeLa or 293T non-silencing control and MOV10 KD cells, and the effect on virus production and infectivity was determined by $p24^{Gag}$ ELISA and TZM-bl assay as previously described. However, similarly to the transfection assay, MOV10 knockdown had no significant effect on HIV-1 virion production (Figure 5.3A) or the infectivity of virus produced (Figure 5.3B). These results verify that natural levels of MOV10 do not impact HIV-1 virus production or infectivity.

5.2.3 Spreading HIV-1 replication is unaffected by silencing of endogenous MOV10

MOV10 was also depleted in the HUT78 T cell line by transducing cells with lentiviral vectors expressing either the non-silencing control or MOV10-specific shRNA as described (section 5.2.1). Again, analysis of HUT78 cell lysates by immunoblotting with an anti-MOV10 antibody revealed that the KD was efficient, whereby MOV10 could not be detected at the protein level (Figure 5.4). As these cells express CD4 and CXCR4, the effect of endogenous MOV10 depletion on multiple rounds of HIV-1 replication was determined. HUT78 non-silencing control and MOV10 KD cells were infected with equal amounts of wild-type HIV-1_{NL4-3} virus and the effect on spreading replication was assessed by determining the concentration of $p24^{Gag}$ in the culture supernatant at days 2, 4, 6 and 8 by $p24^{Gag}$ ELISA. Similar to the observations with single-cycle infectivity assays, silencing of endogenous MOV10 had no effect on spreading HIV-1 replication (Figure 5.4). Therefore, endogenous levels of MOV10 do not restrict the production, infectivity or replication of HIV-1.

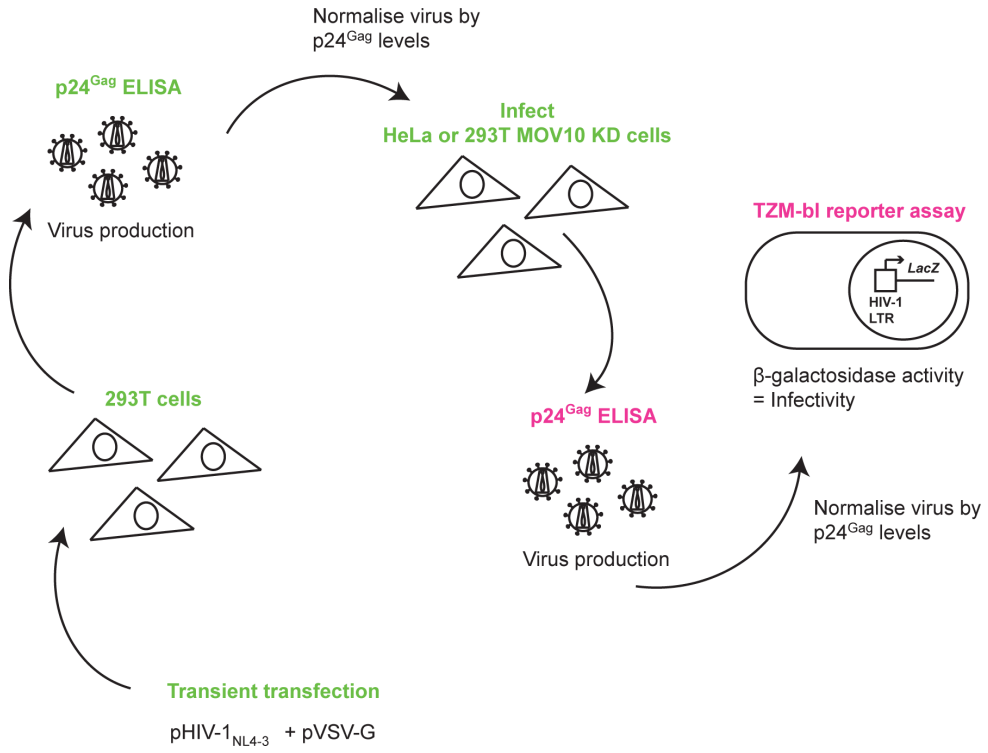


Figure 5.2. Infection assay.

293T cells were co-transfected with pHIV-1_{NL4-3} and pVSV-G to produce VSV-G pseudotyped virus. Virions were quantified by $p24^{Gag}$ ELISA and an equal amount of virus (25 ngs, MOI 0.3) normalised by the $p24^{Gag}$ concentration was used to infect HeLa or 293T non-silencing control and MOV10 KD cells. Approximately 48hrs post-infection virus-containing supernatant was harvested. Virus production was quantified by $p24^{Gag}$ ELISA and virion infectivity was measured by TZM-bl assay as described in chapter 3 Figure 3.1B.

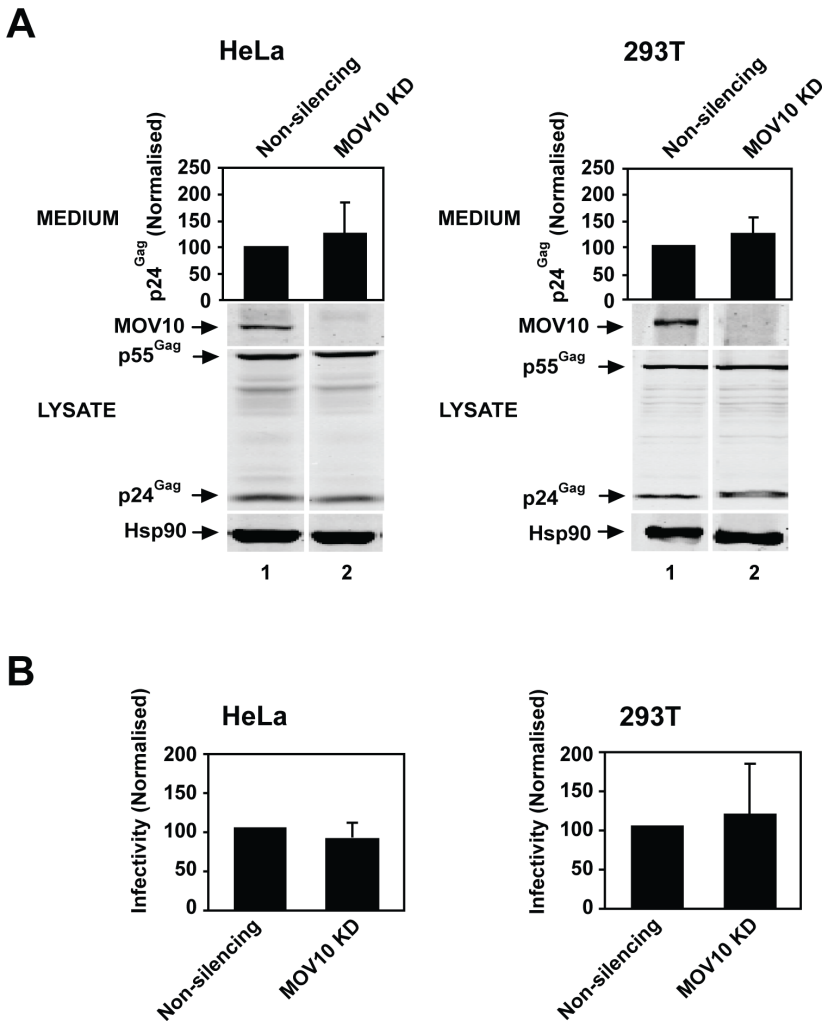


Figure 5.3. Depletion of endogenous MOV10 has no effect on HIV-1 virus production or the infectivity of virions produced (infection assay).

(A) Depletion of endogenous MOV10 has no effect on HIV-1 virus production (infection assay). HeLa or 293T non-silencing control and MOV10 KD cells were infected with VSV-G pseudotyped HIV-1_{NL4-3} virus as described in Figure 5.2. Virus production was quantified by $p24^{Gag}$ ELISA as described in Figure 5.2. Cell lysates were analysed by immunoblotting with anti-MOV10, anti-$p24^{Gag}$ and anti-Hsp90 antibodies. **(B) HIV-1 virion infectivity is unaffected by endogenous MOV10 silencing (infection assay).** The infectivity of equal amounts of virus normalised by the $p24^{Gag}$ concentration from the experiments in panel (A) was measured by TZM-bl assay as described in Figure 5.2. Results are normalised to the non-silencing control, which is set at 100%. Values are the mean ± SD of 7 independent experiments. The data were analysed with an unpaired one-tailed *t* test. (HeLa virus production p = 0.0611, 293T virus production p = 0.2007, HeLa infectivity p = 0.3080 and 293T infectivity p = 0.4812).

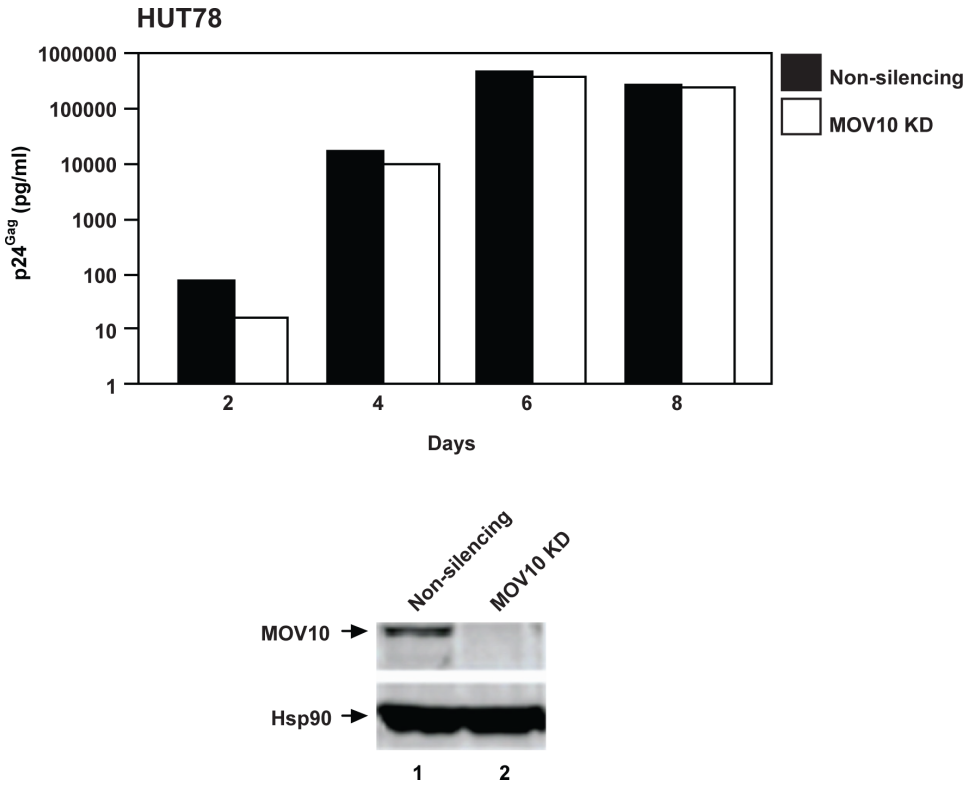


Figure 5.4. Spreading HIV-1 replication is unaffected by silencing of endogenous MOV10.

HUT78 non-silencing control and MOV10 KD T cell lines were produced by transducing cells with lentiviral vectors expressing a non-silencing control shRNA or a MOV10-specific shRNA, respectively. Cell cultures were treated with puromycin to select for successfully transduced cells. HUT78 non-silencing control or MOV10 KD cells were infected with equal amounts of HIV-1_{NL4-3} virus (100 ngs) and passaged every 2 days. Medium was harvested on days 2, 4, 6 and 8 and virus production was quantified as described in chapter 3 Figure 3.1B. Cell lysates were analysed by immunoblotting with anti-MOV10 and anti-Hsp90 antibodies.

5.3 Depletion of endogenous MOV10 has no significant effect on the production of infectious SIVmac, MLV or M-PMV particles

Ectopically overexpressed MOV10 drastically decreases the production of infectious SIVmac and M-PMV particles by over 90% relative to the control, and completely abolishes the production of MLV virions (chapter 3 sections 3.6 and 3.7). Considering that MOV10 KD had no effect on HIV-1 replication, the impact of silencing endogenous MOV10 on the production of infectious SIVmac, MLV and M-PMV particles was also assessed. SIVmac and HIV-1 vectors were produced as previously described (chapter 3 section 3.6) by co-transfecting 293T non-silencing control and MOV10 KD cells with either pSIV3+ and pSIV-RMES4 or p8.91 and pCSGW, respectively, together with pVSV-G. The VSV-G pseudotyped vectors were harvested and equal amounts of vector-containing supernatant was added to 293T cells. Quantification of the percentage of GFP+ cells revealed that similar to wild-type HIV-1 experiments, MOV10 KD had no significant effect on the production of SIVmac particles either (Figure 5.5). MLV and M-PMV virions were also produced as previously described (chapter 3 section 3.7) by co-transfecting 293T non-silencing control and MOV10 KD cells with either pMLV, pMLV-Tat and pVSV-G or pMTΔE and pVSV-G, respectively, and equal amounts of virus-containing supernatant was added to TZM-bl cells. Silencing of endogenous MOV10 had no effect on the production of infectious MLV and M-PMV virions either (Figure 5.5). A role for undetectable levels of residual MOV10 in the KD cells cannot be ruled out, however, these results suggest that, similar to the observations for HIV-1, endogenous levels of MOV10 do not control the replication of SIVmac, MLV or M-PMV.

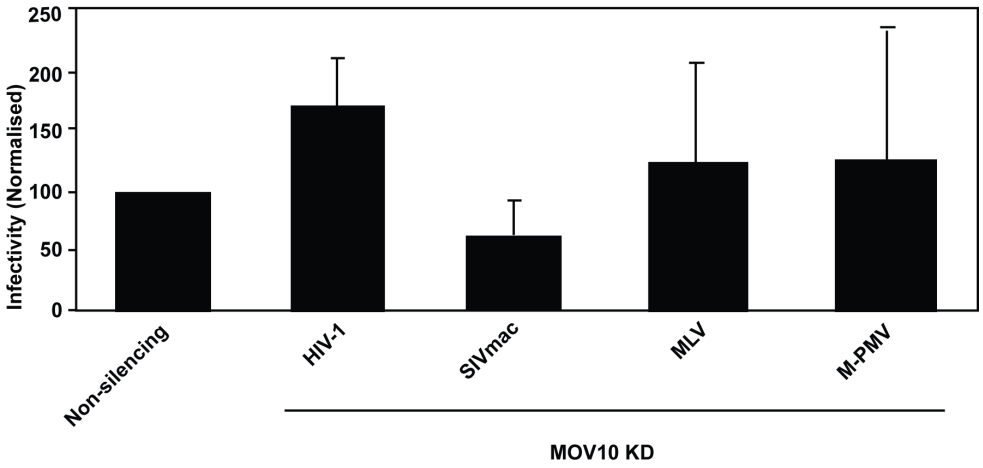


Figure 5.5. Depletion of endogenous MOV10 has no significant effect on the production of infectious SIVmac, MLV or M-PMV particles.

293T non-silencing control and MOV10 KD cells were transfected with plasmids as described in chapter 3 Figure 3.6A, 3.7B and 3.8C for the production of SIVmac, HIV-1, MLV and M-PMV particles. The effect on production of infectious SIVmac and HIV-1 vectors was determined by FACS analysis as described in chapter 3 Figure 3.5C, and for MLV and M-PMV virions this was determined by TZM-bl assay as described in chapter 3 Figure 3.7A and 3.8B. Results are normalised to the non-silencing control, which is set at 100%. A single control bar set at 100% is graphed for simplicity. Values are the mean ± SD of 3 independent experiments. The data were analysed with an unpaired one-tailed *t* test. (HIV-1 p = 0.1358, SIVmac p = 0.1040, MLV p = 0.4907, M- PMV p = 0.4919).

5.4 Spreading HIV-1 replication has no effect on endogenous MOV10 protein abundance

Although A3F and A3G are potent HIV-1 restriction factors their antiviral activity during physiological infection is hampered through targeting of these proteins for proteasomal degradation by the viral accessory protein Vif (Sheehy et al., 2003; Wiegand et al., 2004) (chapter 1 section 1.8.2.8). Similarly, the broad antiviral factor tetherin is antagonised by the primate lentivirus Vpu, Nef and Env proteins resulting in its downregulation and degradation, permitting efficient release of viral particles from the surface of infected cells (Neil et al., 2007; Le Tortorec and Neil, 2009; Zhang et al., 2009)(chapter 1 section 1.8.2.5). More recently, the HIV-2/SIVsmm Vpx protein was also shown to counteract the myeloid cell restriction factor SAMHD1, similarly targeting it for proteasomal degradation and consequently permitting infection of otherwise poorly infected DCs and macrophages (Hrecka et al., 2011; Laguette et al., 2011) (chapter 1 section 1.8.2.6). Ectopically overexpressed MOV10 effectively inhibits the replication of exogenous retroviruses, however, these viruses are refractory to restriction by natural levels of MOV10; therefore, the possibility that viral proteins may antagonise endogenous MOV10 and, therefore, counteract its antiviral activity was investigated.

To test this the HUT78 and CEM-SS T cell lines were infected with equal amounts of wild-type HIV-1_{NL4-3} virus in a spreading replication assay and cell lysates were analysed by immunoblotting with an anti-MOV10 antibody to detect endogenous MOV10 levels. For HUT78 cell lysates, A3G levels were also detected with an anti-A3G antibody as a control for degradation. The results showed that endogenous MOV10 levels were unaffected by HIV-1 replication as similar levels of MOV10 were detected in both infected and mock-infected cells. An exception to this was at day 6 and day 8 for HUT78 and CEM-SS cells, respectively, however this was a consequence of cell death as deduced by the Hsp90 levels (Figure 5.6, lane 6 left panel and lane 8 right panel). Moreover, A3G was degraded as expected by day 4 (Figure 5.6, left panel). Therefore, HIV-1 replication does not degrade endogenous MOV10 protein.

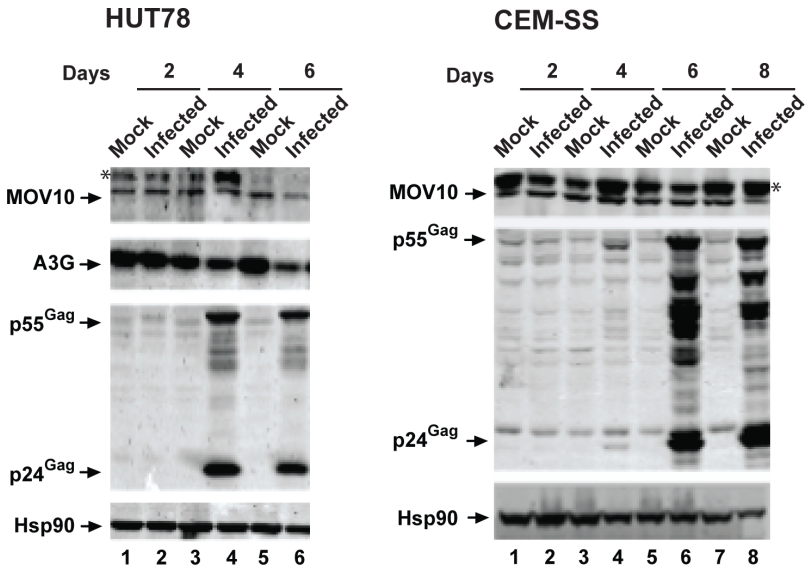


Figure 5.6. Spreading HIV-1 replication has no effect on endogenous MOV10 protein abundance.

HUT78 and CEM-SS T cell lines were mock-infected or infected with equal amounts (100 ngs) of HIV-1_{NL4-3} virus in a spreading replication assay. The cultures were passaged every 2 days and harvested at the indicated days. Cell lysates were analysed by immunoblotting with anti-MOV10, anti-$p24^{Gag}$ and anti-Hsp90 antibodies. For HUT78 cells, A3G protein levels were also detected by immunoblotting with an anti-A3G antibody (* refers to non-specific band).

5.5 Effect of IFNα treatment on endogenous MOV10 protein levels and localisation

5.5.1 IFNα treatment does not stimulate MOV10 protein expression or alter the subcellular localisation of endogenous MOV10 in cell lines

The results presented so far demonstrate that although ectopically overexpressed MOV10 potently decreases the production of infectious retroviral particles, natural levels of MOV10 do not restrict the replication of these retroviruses. Furthermore, MOV10 protein expression is not affected by HIV-1 replication indicating that unlike other antiretroviral factors MOV10 is not degraded by a viral protein, which would otherwise explain the lack of a phenotype in MOV10 KD studies. An alternative theory is that during physiological infection MOV10 protein expression may be stimulated to levels sufficient for the restriction of retroviruses, mimicking the observations with MOV10 overexpression. The family of Type I IFN cytokines are produced during early infection and are critical for the onset of an antiviral innate and adaptive immune response. Interestingly, IFNα treatment induces expression of the antiviral APOBEC3 proteins, tetherin and SAMHD1 and, therefore, the effect of IFNα treatment on MOV10 protein expression was also determined.

293T or the CEM, HUT78 and Jurkat T cell lines were cultured in the presence of IFNα for approximately 24hrs, following which cell lysates were analysed by immunoblotting with anti-MOV10 and anti-Hsp90 antibodies. As a control for IFNα treatment, levels of the known ISG TRIM22 were also detected with an anti-TRIM22 antibody. TRIM22 could not be detected in 293T cells, however, IFNα treatment notably stimulated TRIM22 protein expression in CEM, HUT78 and Jurkat cells compared to the mock-treated cells (Figure 5.7A). In contrast, MOV10 protein expression remained unchanged in IFNα-treated and mock-treated cells for all cell lines tested (Figure 5.7A). The effect of IFNα treatment on MOV10 protein expression was also investigated in a time-course to determine the kinetics of this reaction and ensure that MOV10 protein expression was not stimulated earlier or later than 24hrs. For this HeLa or 293T cells were cultured in the presence of IFNα for 12hrs, 24hrs, 36hrs and 48hrs, and cell lysates were analysed by immunoblotting with anti-MOV10 and anti-Hsp90 antibodies. MOV10 protein expression was not induced at any of these time points with IFNα treatment in HeLa or 293T cells relative to the mock-treated cells (Figure 5.7B). Therefore, MOV10 protein expression is not induced by IFNα in cell lines.

Alternatively, IFNα treatment may alter the subcellular localisation of MOV10 in a way that could potentially enhance its antiviral activity. To test this HeLa cells were cultured in the presence of IFNα for 24hrs following which cells were fixed and analysed by immunofluorescence using an anti-MOV10 antibody. However, the subcellular distribution of MOV10 in IFNα-treated and mock-treated cells was similar, whereby MOV10 was cytoplasmically diffuse and localised to P bodies (Figure 5.7C). Therefore, IFNα treatment has no effect on endogenous MOV10 subcellular distribution either.

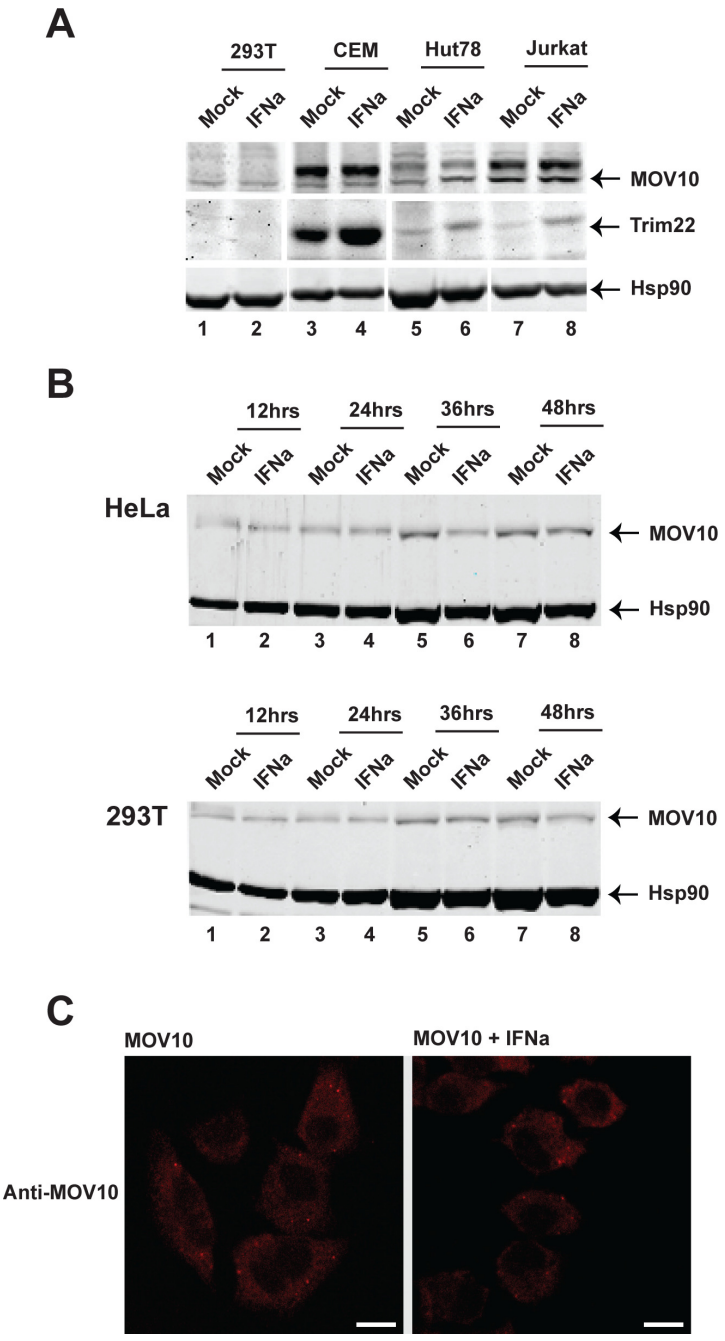


Figure 5.7. IFNα treatment does not stimulate MOV10 protein expression or alter the subcellular localisation of endogenous MOV10 in cell lines.

(A) IFNα treatment does not stimulate MOV10 protein expression in cell lines. 293T, CEM, HUT78 and Jurkat cells were treated with IFNα for approximately 24hrs. Cell lysates were analysed by immunoblotting with anti-MOV10, anti-TRIM22 and anti-Hsp90 antibodies. **(B) MOV10 protein abundance is not induced by IFNα treatment in a time-course.** HeLa and 293T cells were treated with IFNα for 12hrs, 24hrs, 36hrs and 48hrs. Cell lysates were analysed by immunoblotting with anti-MOV10 and anti-Hsp90 antibodies. **(C) IFNα treatment does not alter the subcellular localisation of endogenous MOV10 in cell lines.** HeLa cells were treated with IFNα for approximately 24hrs, fixed in 4% PFA and stained with an anti-MOV10 primary antibody and appropriate Alexa Fluor conjugated secondary antibody. Coverslips were mounted onto slides and analysed by immunofluorescence. Scale bar = 10μm.

5.5.2 MOV10 protein expression is not induced by IFNα treatment in primary cells

IFNα treatment does not induce MOV10 protein expression in a panel of cell lines, however, the IFNα-responsiveness of cell lines may vary from that of primary cells. Therefore, the effect of IFNα treatment on MOV10 protein expression in primary CD4+ T cells, monocyte-derived DCs (MDDCs) and MDMs was assessed. Naïve CD4+ T cells were isolated from 2 donors and ex vivo analysis of cell lysates by immunoblotting with anti-MOV10 and anti-Hsp90 antibodies revealed that MOV10 was expressed in these cells (Figure 5.8A, donor 2 lane 1). The lack of detection in donor 1 and only faint MOV10 protein levels in donor 2 is likely attributed to low cell numbers as deduced by Hsp90 levels. Activation of naïve CD4+ T cells with anti-CD3/CD28 or interleukin-2 (IL-2)/phytohaemagglutinin (PHA) stimulation resulted in the detection of higher MOV10 levels with the latter treatment after 5 days (Figure 5.8A, both donors lane 5) due to higher levels of cell proliferation with IL-2/PHA stimulation compared to anti CD3/CD28 stimulation as deduced from the Hsp90 levels (Figure 5.8A, both donors compare lanes 4 and 5). IL-2/PHA pre-stimulated CD4+ T cells were cultured in the presence of IFNα for 24hrs or 48hrs and cell lysates were analysed by immunoblotting with anti-MOV10 and anti-Hsp90 antibodies. Results showed that at both time-points MOV10 protein abundance remained unchanged relative to the mock-treated cells (Figure 5.8B) confirming that MOV10 protein expression is not induced by IFNα treatment in primary CD4+ T cells.

MDDCs and MDMs were cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), or GM-CSF alone for 5 days, respectively, following which cells were treated with IFNα. Cell lysates were analysed by immunoblotting with anti-MOV10 and anti-Hsp90 antibodies, as well as an antibody against A3G as a positive control for IFNα-mediated induction in MDDCs. Similar to primary CD4+ T cells, IFNα treatment of MDDCs for 6hrs and 30hrs, and MDMs for 30hrs had no effect on MOV10 protein expression compared to mock-treated cells (Figure 5.8C). These results demonstrate that IFNα treatment does not stimulate MOV10 protein expression in a range of cell lines or primary cells, including CD4+ T cells and macrophages, which are the natural targets for HIV-1 infection.

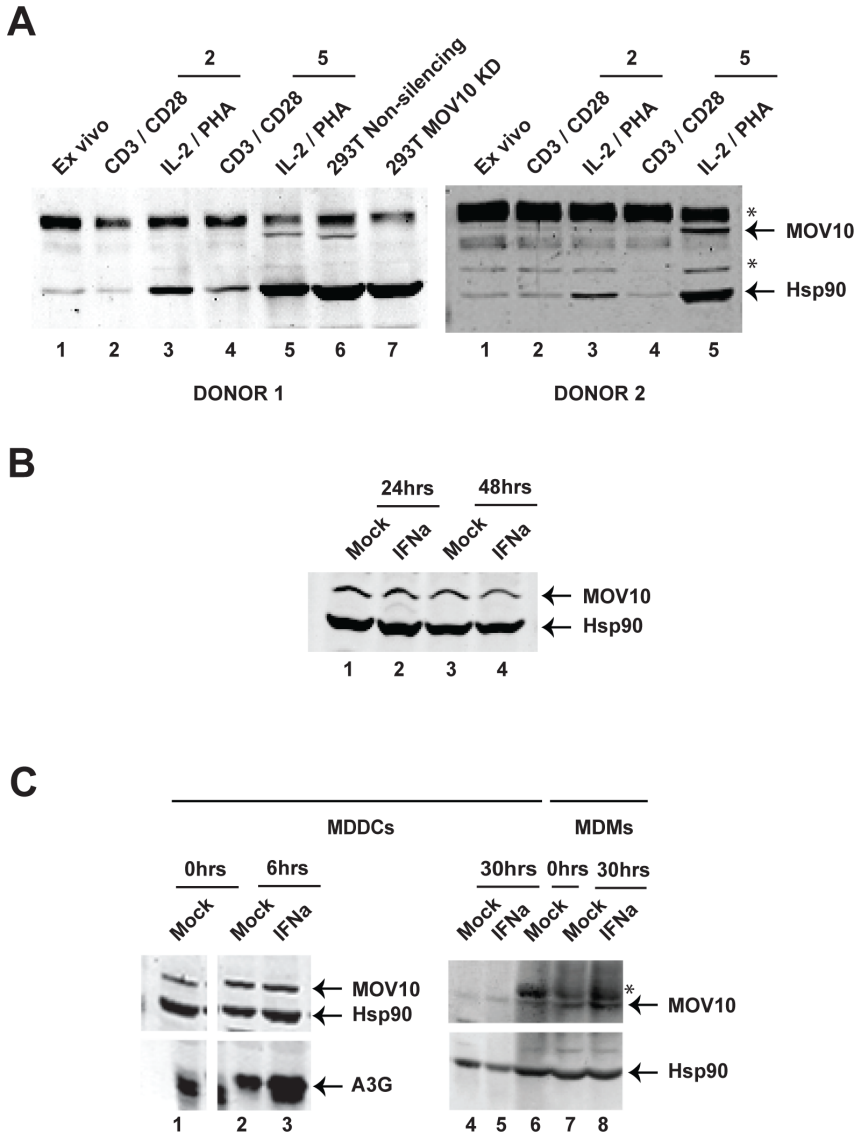


Figure 5.8. MOV10 protein expression is not induced by IFNα treatment in primary cells.

(A) MOV10 is expressed in primary CD4+ T cells. Naïve CD4+ T cells were isolated from peripheral blood mononuclear cells (PBMCs) from two donors. Cell lysates were analysed ex vivo by immunoblotting with anti-MOV10 and anti-Hsp90 antibodies. Cells were also plated and activated with anti-CD3/CD28 or interleukin-2 (IL-2)/phytohaemagglutinin (PHA) stimulation. Cells were harvested at days 2 and 5 and lysates were analysed by immunoblotting with anti-MOV10 and anti-Hsp90 antibodies. 293T non-silencing control and MOV10 KD cell lysates were included in the analysis as a marker for the MOV10 band. **(B) MOV10 protein expression is not induced by IFNα treatment in primary CD4+ T cells.** CD4+ T cells pre-stimulated with IL-2/PHA for 2 days were either mock-treated or cultured in the presence of IFNα for 24hrs or 48hrs. Cell lysates were analysed by immunoblotting with anti-MOV10 and anti-Hsp90 antibodies. **(C) MOV10 protein expression is not induced by IFNα treatment in monocyte-derived dendritic cells (MDDCs) and macrophages (MDMs).** Primary monocytes were differentiated into MDDCs and MDMs by treating cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), or GM-CSF alone for 5 days, respectively. Subsequently, cells were cultured in the absence or presence of IFNα for the indicated times. Cell lysates were analysed by immunoblotting with anti-MOV10 and anti-Hsp90 antibodies, and also anti-A3G for the MDDCs (* refers to non-specific band).

5.6 Effect of depleting endogenous MOV10 on the replication of endogenous retroelements

5.6.1 Silencing of endogenous MOV10 enhances the retrotransposition of LTR and non-LTR endogenous retroelements

The results so far demonstrate that endogenous levels of MOV10 do not control the replication of exogenous retroviruses. Ectopically overexpressed human MOV10 also potently suppresses the replication of the mouse ERV IAP and human retrotransposons LINE-1 and Alu (chapter 3 section 3.8). To determine whether natural levels of MOV10 suppress the replication of these endogenous retroelements, the effect of endogenous MOV10 silencing on IAP, LINE-1 and Alu retrotransposition was determined. HeLa non-silencing control and MOV10 KD cells were transfected as previously described (chapter 3 section 3.8) with pGL3-IAP92L23neo^{TNF} (pIAP), pJM101/L1.3 (pLINE-1) or pAlu-neo^{Tet} (pAlu) and pORF2p, and cell cultures were G418 selected. Interestingly, depletion of endogenous MOV10 significantly enhanced IAP, LINE-1 and Alu retrotransposition by 2-fold, 4-fold and 5-fold, respectively (Figure 5.9A). Again to assess whether MOV10 KD affected the expression or selection of *neo* directly, or alternatively, increased the transfection efficiency of these cells, HeLa non-silencing control and MOV10 KD cells were transfected with pcDNA3.1-*neo* and the retrotransposition assay was performed as described. A similar number of colonies were counted for the non-silencing control and MOV10 KD cells confirming that silencing of endogenous MOV10 has no effect on *neo* expression, selection or the transfection efficiency of cells (Figure 5.9B). Therefore, natural levels of MOV10 control the replication of LTR and non-LTR endogenous retroelements.

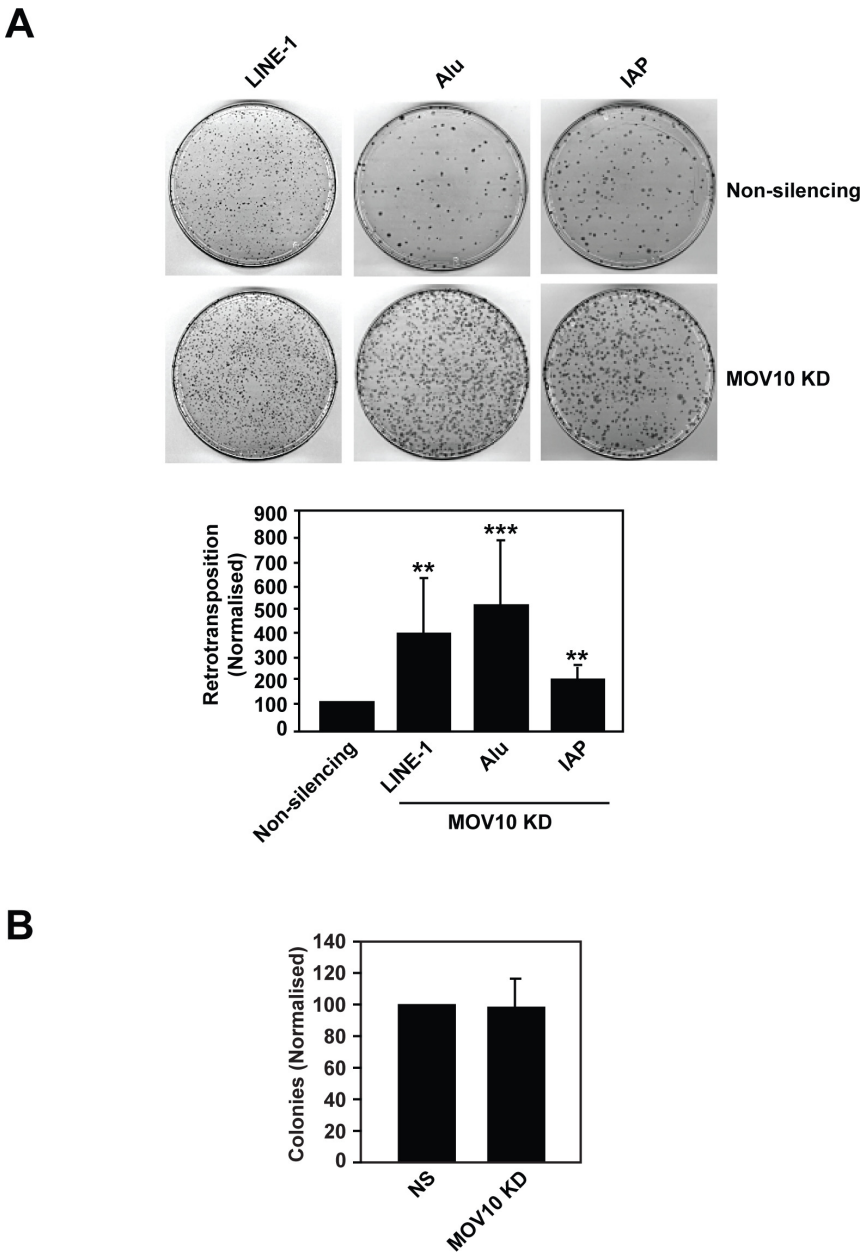


Figure 5.9. Silencing of endogenous MOV10 enhances the retrotransposition of LTR and non-LTR endogenous retroelements.

(A) Silencing of endogenous MOV10 enhances the retrotransposition of LTR and non-LTR endogenous retroelements. HeLa non-silencing control or MOV10 KD cells were transfected with pGL3-IAP92L23neo^{TNF} (pIAP), pJM101/L1.3 (pLINE-1) or pAlu-neo^{Tet} (pAlu) as described in chapter 3 Figure 3.10A. The retrotransposition frequency was determined by G418 selection of the cultures as described in chapter 3 Figure 3.9. **(B) Endogenous MOV10 KD has no direct effect on *neo* expression, selection or the transfection efficiency of cells.** HeLa non-silencing control and MOV10 KD cells were transfected with pcDNA3.1-*neo* as described chapter 3 Figure 3.10B. The retrotransposition frequency was determined by G418 selection of the cultures as described in chapter 3 Figure 3.9. Results are normalised to the non-silencing control, which is set at 100%. For panel (A) a single control bar set at 100% is graphed for simplicity. Values are the mean ± SD of 3 independent experiments. The data were analysed with an unpaired one-tailed *t* test. (LINE-1 **p ≤ 0.0056, Alu ***p ≤ 0.0005, IAP **p ≤ 0.0096).

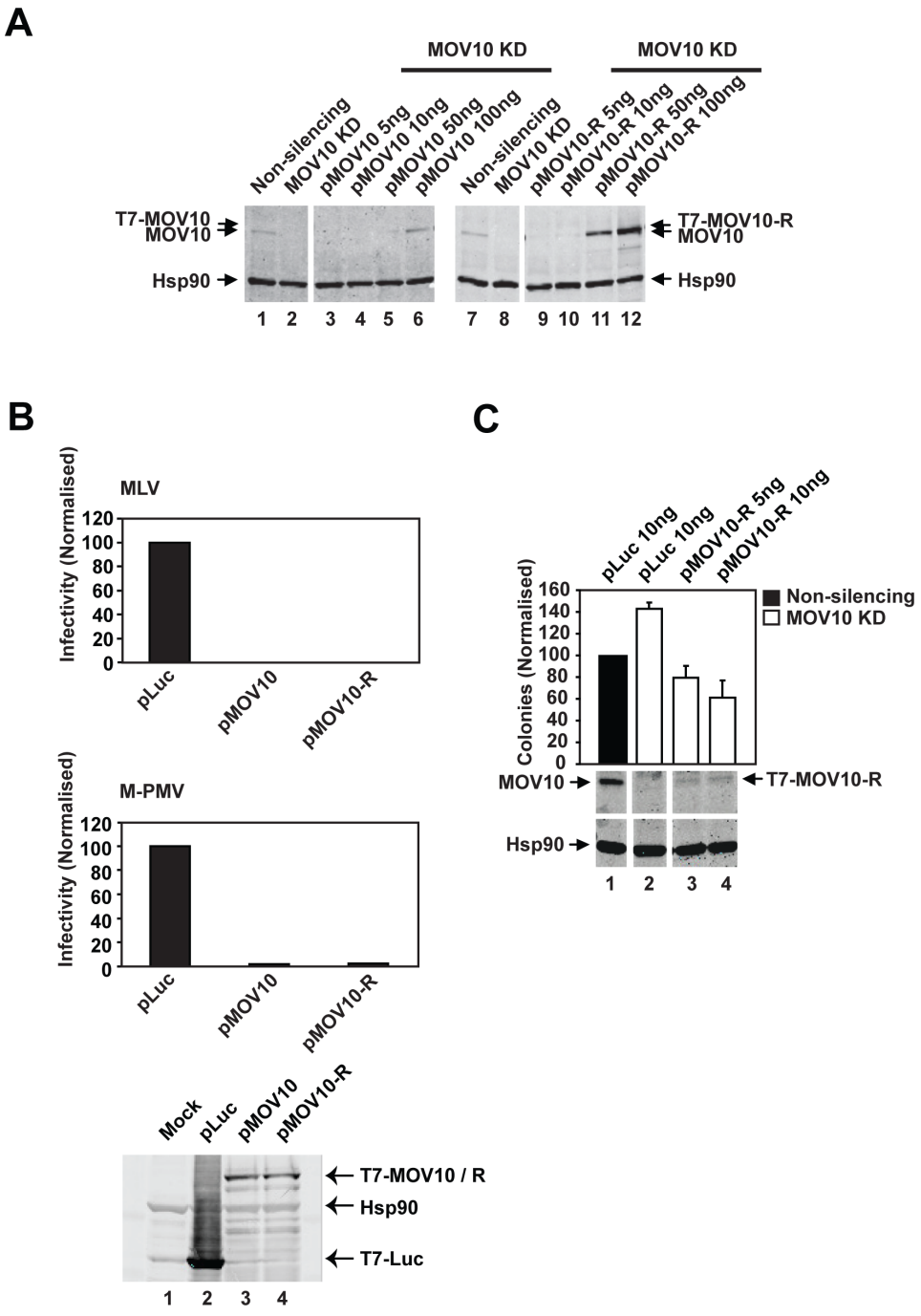
5.6.2 Restoration of MOV10 expression in MOV10 KD cells rescues the control of LINE-1 retrotransposition

To ensure that the enhanced retrotransposition of endogenous retroelements was attributed specifically to the depletion of endogenous MOV10 and not an unanticipated off-target effect, a silencing resistant version of MOV10 was generated by introducing silent mutations into the shRNA target sequence. This was cloned into the pT7 plasmid generating pT7-MOV10-R (pMOV10-R). To test the shRNA-resistance of pMOV10-R, HeLa MOV10 KD cells were transfected with increasing concentrations of either sensitive pMOV10 or resistant pMOV10-R, and cell lysates were analysed by immunoblotting with anti-MOV10, anti-T7 and anti-Hsp90 antibodies. Results showed that the abundance of MOV10-R was elevated in comparison to wild-type MOV10 (Figure 5.10A, compare lanes 5 and 6 with 11 and 12), verifying the resistance of this construct to the MOV10-specific shRNA expressed in MOV10 KD cells. The effect of ectopically overexpressed MOV10-R on the production of infectious MLV and M-PMV virions was also examined to assess the antiviral function of MOV10-R relative to wild-type MOV10. Analysis of cell lysates by immunoblotting with anti-T7 and anti-Hsp90 antibodies demonstrated MOV10-R and wild-type MOV10 expression levels to be similar (Figure 5.10B, lower panel) and, moreover, ectopically overexpressed MOV10-R decreased the production of infectious MLV and M-PMV as effectively as wild-type MOV10 (Figure 5.10B).

Subsequently, the effect of restoring MOV10 expression in MOV10 KD cells on LINE-1 retrotransposition was determined by transfecting HeLa non-silencing control or MOV10 KD cells with pJM101/L1.3 (pLINE-1), and transfecting MOV10 KD cells with increasing concentrations of pMOV10-R or the pT7-Luc (pLuc) control. The non-silencing control cells were also transfected with pLuc to ensure equivalent amounts of DNA in all transfections. Results showed an expected increase in LINE-1 retrotransposition with MOV10 KD relative to the non-silencing control cells (Figure 5.10C, compare lanes 1 and 2). Moreover, expression of pMOV10-R in MOV10 KD cells restored the control of LINE-1 retrotransposition (Figure 5.10C, lanes 3 and 4). These results confirm that depletion of specifically endogenous MOV10, and not an off-target effect, enhances LINE-1 retrotransposition.

Figure 5.10. Restoration of MOV10 expression in MOV10 KD cells rescues the control of LINE-1 retrotransposition.

(A) Testing the shRNA-resistance of pT7-MOV10-R. HeLa MOV10 KD cells were transfected with increasing concentrations of pT7-MOV10 (pMOV10) or pT7-MOV10-R (pMOV10-R) as indicated. Cell lysates were analysed by immunoblotting with anti-MOV10, anti-T7 and anti-Hsp90 antibodies**. (B) The antiviral activity of MOV10-R is similar to wild-type MOV10.** 293T cells were co-transfected with plasmids for the production of MLV and M-PMV virions as described in chapter 3 Figure 3.7B and 3.8C together with wild-type pT7-MOV10 (pMOV10) or pT7-MOV10-R (pMOV10-R) (0.5 μg). The effect on production of infectious virions was determined by TZM-bl assay as described in chapter 3 Figure 3.7A and 3.7B. Cell lysates were analysed by immunoblotting with anti-T7 and anti-Hsp90 antibodies. **(C) Restoration of MOV10 expression in MOV10 KD cells rescues the control of LINE-1 retrotransposition.** HeLa non-silencing control and MOV10 KD cells were transfected with pJM101/L1.3 (pLINE-1) as described in chapter 3 Figure 3.10A, and MOV10 KD cells were also transfected with pT7-MOV10-R (pMOV10-R) or the pT7-Luc (pLuc) control at the indicated concentrations. Non-silencing control cells were transfected with pLuc to ensure equal amounts of DNA in all transfections. Cells were harvested and lysed approximately 48hrs post-transfection and analysed by immunoblotting with anti-T7 and anti-Hsp90 antibodies. The retrotransposition frequency was determined by G418 selection of the cultures as described in chapter 3 Figure 3.9. Results for panels (B) and (C) are normalised to the relative controls, which are set at 100%. For panel (C) values are the mean ± SD of 3 independent experiments.


A
MOV10 KD
MOV10 KD
Non-silencing
MOV10 KD
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pMOV10 10ng
pMOV10 50ng
pMOV10 100ng
Non-silencing
MOV10 KD
pMOV10-R 5ng
pMOV10-R 10ng
pMOV10-R 50ng
pMOV10-R 100ng
T7-MOV10
MOV10
Hsp90
T7-MOV10-R
MOV10
Hsp90
1 2 3 4 5 6 7 8 9 10 11 12
B
MLV
Infectivity (Normalised)
120
100
80
60
40
20
0
pLuc
pMOV10
pMOV10-R
M-PMV
Infectivity (Normalised)
120
100
80
60
40
20
0
pLuc
pMOV10
pMOV10-R
Mock
pLuc
pMOV10
pMOV10-R
T7-MOV10 / R
Hsp90
T7-Luc
1 2 3 4
C
pLuc 10ng
pLuc 10ng
pMOV10-R 5ng
pMOV10-R 10ng
Colonies (Normalised)
160
140
120
100
80
60
40
20
0
Non-silencing
MOV10 KD
MOV10
T7-MOV10-R
Hsp90
1 2 3 4

5.7 Requirement for MOV10 in small RNA-mediated post-transcriptional RNA silencing pathways

5.7.1 MOV10 is not necessary for miRNA-mediated translation repression

To better understand the mechanism by which natural levels of MOV10 control the retrotransposition of endogenous retroelements, the cellular associations of MOV10 were explored. Meister et al identified an interaction between MOV10 and members of the AGO protein family, and also proposed a role for MOV10 in miRNA-mediated cleavage of a reporter mRNA (Meister et al., 2005). To determine whether MOV10 is required for miRNA-mediated translation repression and, therefore, also establish whether this is a potential mechanism by which endogenous MOV10 suppresses retrotransposition, an established firefly Luc reporter assay was adopted (Lytle et al., 2007). The wild-type (WT) reporter construct expresses firefly Luc with a 3' UTR containing four endogenous let-7 miRNA binding sites from a CMV promoter, and in the case of the mutant reporter construct these endogenous let-7 miRNA binding sites are mutated (Lytle et al., 2007) (Figure 5.11A). Therefore, endogenous let-7 miRNA will bind and repress firefly Luc expression from the WT reporter construct, but not the mutant reporter construct. HeLa non-silencing control and MOV10 KD cells were transfected with the WT or mutant reporter constructs, together with a plasmid expressing renilla Luc (pRenilla) as a control for transfection efficiency (Figure 5.11A). Cell lysates were analysed with a dual Luc reporter assay to measure relative firefly and renilla Luc activities, and firefly Luc activity was normalised to renilla Luc activity to normalise for small changes in transfection efficiency. As expected, Luc activity was repressed by approximately 5-fold in the case of the WT reporter construct relative to the mutant construct for non-silencing control cells (Figure 5.11B). Interestingly, this level of repression was maintained in the MOV10 KD cells indicating that MOV10 is not essential for endogenous let-7 miRNA-mediated repression of a reporter construct in HeLa cells (Figure 5.11B).

DICER is an RNase III enzyme essential for miRNA and siRNA biogenesis (chapter 1 section 1.12). As a control for this assay, HeLa cells were transfected with non-silencing control or DICER-1 specific siRNAs, which achieved an approximately 70% KD in DICER-1 transcript levels as determined by qPCR (Figure 5.11C, right panel). Non-silencing control and DICER-1 KD cells were co-transfected with the WT or mutant firefly Luc reporter constructs and pRenilla. Relative firefly and renilla Luc

activities were determined and normalised as described, and as expected the results showed a decrease in Luc activity with the WT reporter construct relative to the mutant reporter construct for non-silencing cells. Moreover, confirming the validity of this approach, the partial DICER-1 knockdown resulted in moderate derepression of Luc activity compared to that observed for the non-silencing control cells (Figure 5.11C, left panel). Therefore, unlike DICER-1, MOV10 is not essential for miRNA-mediated translation repression pathways in cultured cells.

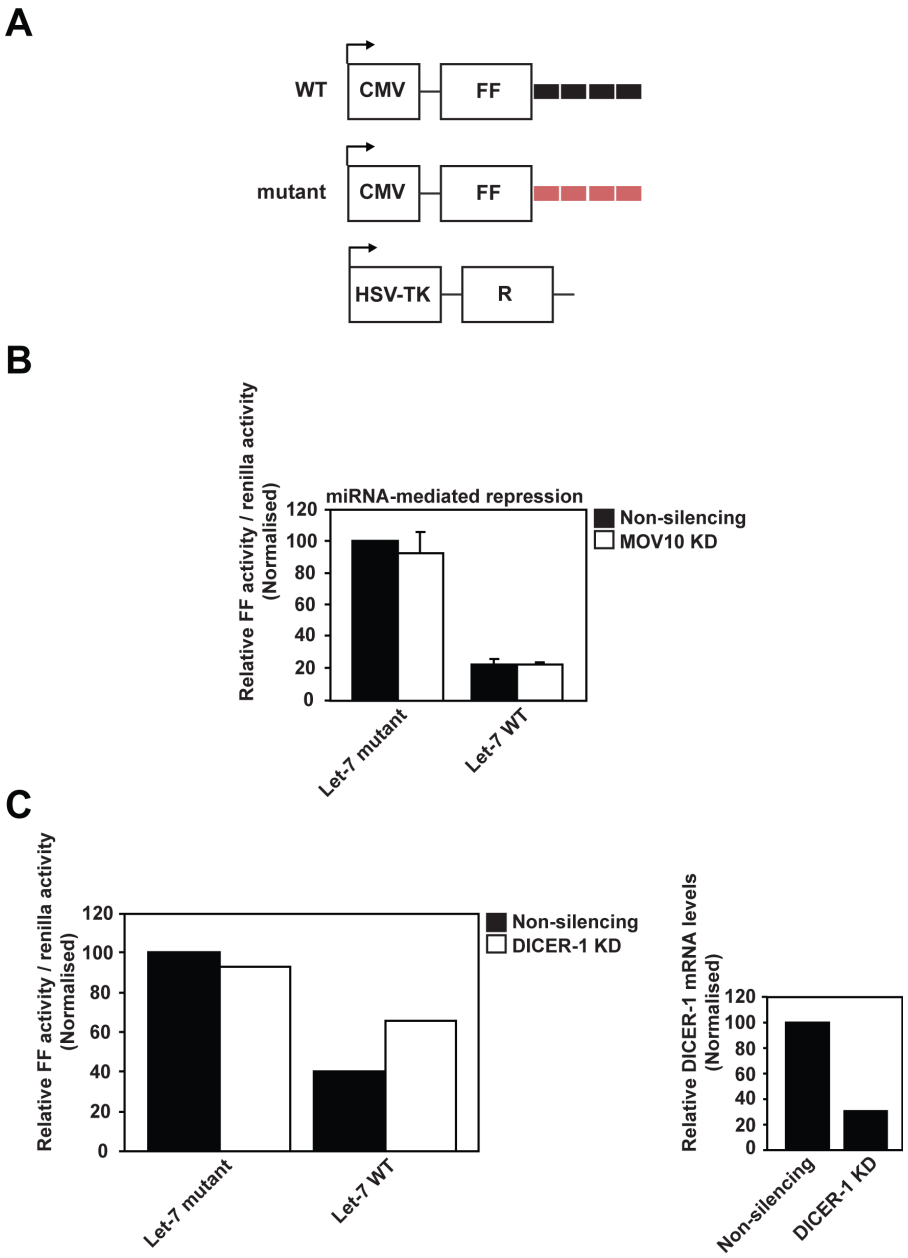

Figure 5.11. MOV10 is not necessary for miRNA-mediated translation repression.

(A) Firefly Luc reporter constructs. The wild-type (WT) construct is driven by a CMV promoter and encodes firefly Luc with four endogenous let-7 miRNA binding sites. The mutant construct is driven by a CMV promoter and encodes firefly Luc with mutated endogenous let-7 miRNA binding sites. The renilla construct is expressed from a herpes simplex virus thymidine kinase (HSV-TK) promoter. **(B) MOV10 is not necessary for miRNA-mediated translation repression.** HeLa non-silencing control and MOV10 KD cells were co-transfected with the WT or mutant firefly Luc reporter constructs (0.1 μg) together with the renilla construct (0.1 μg). Approximately 24hrs post-transfection cells were lysed and the relative firefly and renilla Luc activities were measured using a dual-Luc reporter assay system. Firefly Luc activity was normalised to renilla Luc activity. **(C) Knockdown of DICER-1 relieves miRNA-mediated translation repression.** HeLa cells were transfected with non-silencing control or DICER-1-specific siRNAs to produce HeLa non-silencing control and DICER-1 KD cells, respectively. RNA was extracted from these cells and cDNA was synthesised. DICER-1 mRNA levels were measured by qPCR (right panel). HeLa non-silencing control and DICER-1 KD cells were co-transfected with the WT or mutant firefly constructs (0.1 μg) together with the renilla construct (0.1 μg) and relative firefly and renilla Luc activities were measured as described in panel (B). Results in panels (B) and (C) are normalised to the non-silencing control, which is set at 100%. For panel (B) values are the mean ± SD of 3 independent experiments.

5.7.2 MOV10 is not necessary for miRNA-mediated mRNA cleavage

Although MOV10 associates with the AGO1 and AGO2 proteins, it is not essential for miRNA-mediated translation repression of a reporter construct in HeLa cells. However, MOV10 has been reported to be necessary for miRNA-mediated mRNA cleavage (Meister et al., 2005). Therefore, to confirm whether MOV10 is indeed essential for the miRNA-mediated RNA cleavage pathway and, therefore, also explore this is a potential mechanism by which endogenous MOV10 controls retrotransposition, an established renilla Luc reporter assay was used (Johnston et al., 2010). A wild-type (WT) reporter construct expressing renilla Luc with three 100% complementary endogenous let-7 miRNA binding sites from under a CMV promoter, and a mutant reporter construct encoding renilla Luc with mutated endogenous let-7 miRNA binding sites (Figure 5.12A) were transfected into HeLa non-silencing control or MOV10 KD cells. Both the WT and mutant constructs also expressed firefly Luc from a second downstream promoter as a control for transfection efficiency (Figure 5.12A). For this assay, endogenous let-7 miRNA will cleave the WT reporter construct due to 100% complementary binding sites. Relative renilla and firefly Luc activities were measured using a dual Luc reporter assay as described, and renilla Luc activity was normalised to firefly Luc activity to control for small changes in transfection efficiency. As expected, Luc activity was decreased drastically by approximately 50-fold in the case of the WT reporter construct compared to the mutant reporter construct for non-silencing control cells (Figure 5.12B). However, similar to the observation for translation repression, Luc activity was reduced to similar levels in MOV10 KD cells as well (Figure 5.12B). Therefore, MOV10 is not essential for miRNA-mediated cleavage of a reporter mRNA in cultured cells.

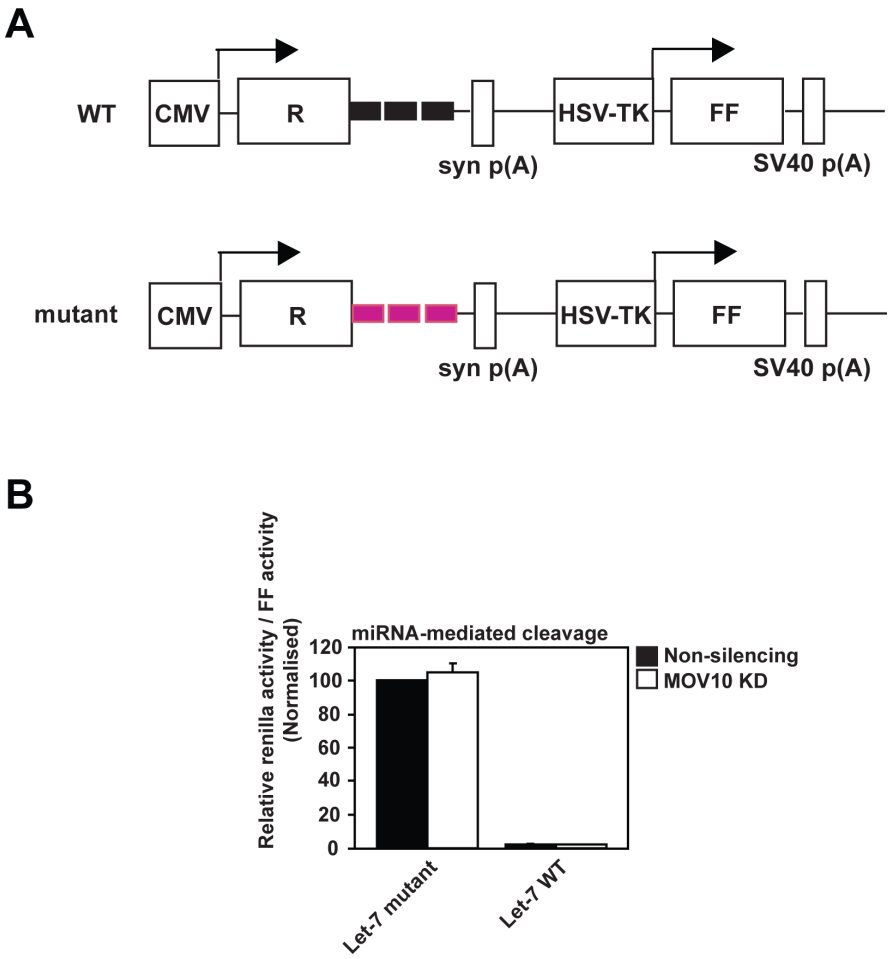

Figure 5.12. MOV10 is not necessary for miRNA-mediated mRNA cleavage.

(A) Renilla Luc reporter constructs. The wild-type (WT) construct is driven by a CMV promoter and encodes renilla Luc with three perfectly complementary endogenous let-7 miRNA binding sites. The mutant construct also contains the CMV promoter, however, encodes renilla Luc with three mutated endogenous let-7 miRNA binding sites. Both the WT and mutant constructs also encode firefly Luc, which is expressed from a second HSV-TK promoter. **(B) MOV10 is not necessary for miRNA-mediated mRNA cleavage.** HeLa non-silencing control and MOV10 KD cells were transfected with either the WT or mutant renilla Luc reporter constructs (0.1 μg). Cells were lysed approximately 24hrs post-transfection and relative renilla and firefly Luc activities were measured with a dual Luc reporter assay system. Renilla Luc activity was normalised to firefly Luc activity. Results are normalised to the non-silencing control, which is set at 100%. Values are the mean ± SD of 3 independent experiments.

5.7.3 Endogenous MOV10 silencing has no effect on P bodies

MOV10 interacts with AGO1 and AGO2 proteins, which are core constituents of the RISC, and also interacts with members of the APOBEC3 family of proteins (Meister et al., 2005; Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). Moreover, MOV10 co-localises with these factors in cytoplasmic mRNA P bodies (Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). Interestingly, miRNA or siRNA-mediated post-transcriptional RNA silencing pathways as well as the APOBEC3 proteins have also been implicated in the negative regulation of endogenous retroelements (chapter 1 sections 1.8.2.9, 1.8.2.10 and 1.12.5). Localisation to P bodies appears to be the common denominator between these various functions and pathways. To determine whether depletion of endogenous MOV10 facilitates retrotransposition due to alterations in P bodies, for example, their depletion, the effect of MOV10 silencing on P bodies was investigated. HeLa non-silencing control and MOV10 KD cells were fixed and analysed by immunofluorescence using antibodies against the P body markers DDX6 and GE1. Results showed no effect of MOV10 KD on both DDX6 and GE1 P bodies compared to the non-silencing control cells (Figure 5.13). This result confirms that the increase in retrotransposition observed with endogenous MOV10 silencing is not attributed to an effect on P bodies, as P bodies remain unchanged in the absence of MOV10.

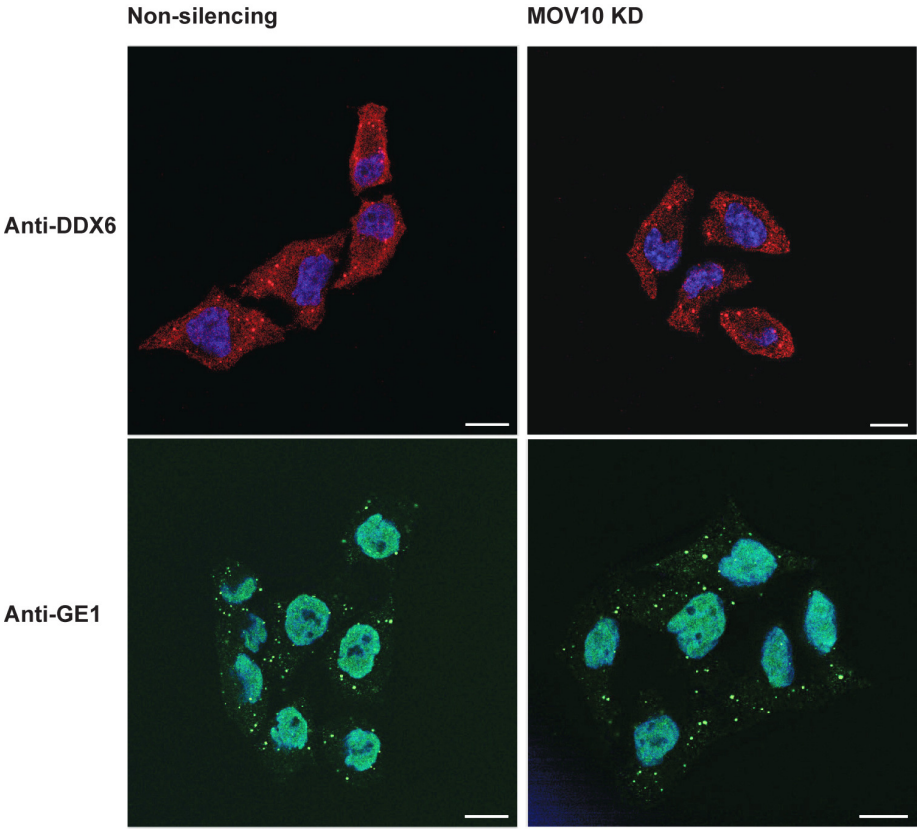

Figure 5.13. Endogenous MOV10 silencing has no effect on P bodies.

HeLa non-silencing and MOV10 KD cells were fixed in 4% PFA and stained with anti-DDX6 and anti-GE1 primary antibodies, followed by appropriate Alexa Fluor conjugated secondary antibodies and DAPI. Coverslips were mounted onto slides and analysed by immunofluorescence. Scale bar = 10µm.

5.8 MOV10 relationship with APOBEC3 proteins

5.8.1 MOV10 is dispensable for suppression of LINE-1 retrotransposition by members of the APOBEC3 family

Overexpression of all APOBEC3 proteins variably restricts the retrotransposition of LINE-1 and also Alu retrotransposons, with A3A and A3B being the most potent members (Bogerd et al., 2006) (chapter 1 section 1.8.2.10). Moreover, silencing of endogenous A3B enhances LINE-1 replication in HeLa and embryonic stem cells (Wissing et al., 2011). As endogenous levels of MOV10 suppress retrotransposition and MOV10 interacts with members of the APOBEC3 family (Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008) the functional relevance of this association for APOBEC3 anti-retrotransposon activity was explored. The effect of endogenous MOV10 depletion on APOBEC3-mediated restriction of LINE-1 retrotransposition was assessed by co-transfecting HeLa non-silencing control and MOV10 KD cells with pJM101/L1.3 (pLINE-1) and pCMV4-A3A-HA (pA3A), pCMV4-A3B-HA (pA3B), pCMV4-A3G-HA (pA3G) or the pCMV4-GFP-HA (pGFP) control (Bishop et al., 2004). The retrotransposition assay was performed as described and enumeration of colonies revealed that ectopically overexpressed A3A effectively inhibited LINE-1 retrotransposition both in the presence and absence of MOV10 (Figure 5.14A). Additionally, overexpression of A3B and A3G suppressed LINE-1 retrotransposition by approximately 70% and 50%, respectively, in the non-silencing control cells relative to the pGFP control, and this level of inhibition was maintained in the MOV10 KD cells (Figure 5.14A). Therefore, ectopically overexpressed A3A, A3B and A3G do not depend on MOV10 for restriction of LINE-1 retrotransposition. On the other hand, as the majority of APOBEC3 transcripts, except A3B and A3C, cannot be detected in HeLa cells, endogenous MOV10 likely controls the retrotransposition of endogenous retroelements independently of APOBEC3 proteins as well.

5.8.2 MOV10 is not required for A3G antiviral activity

Similarly, the requirement of MOV10 for A3G antiviral activity against HIV-1 was also tested by co-transfecting HeLa non-silencing control and MOV10 KD cells with a plasmid expressing a vif-deficient HIV-1 IIIB provirus (pHIV-1_{IIIB}/ΔVif) (Bishop et al., 2004) and either pA3G or the pGFP control. The infectivity of HIV-1 virions was reduced by approximately 200-fold with A3G overexpression and, similarly to LINE-1

retrotransposition, this potent reduction was sustained in the MOV10 KD cells, indicating that MOV10 is not required for A3G antiviral activity either.

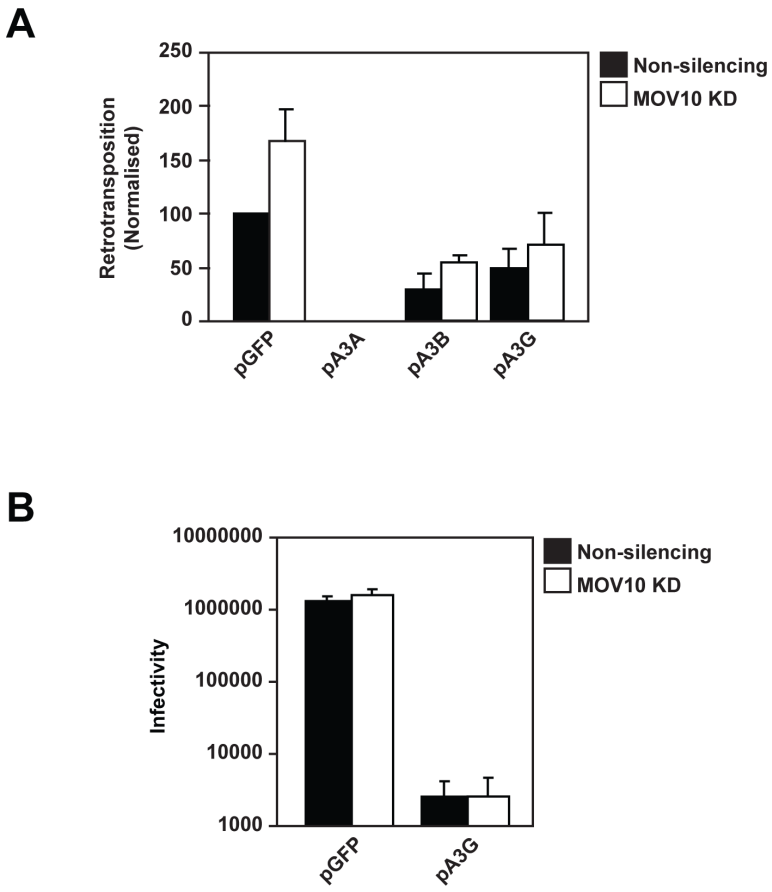

Figure 5.14. MOV10 is dispensable for the restriction of LINE-1 and HIV-1 replication by APOBEC3 proteins.

(A) MOV10 is dispensable for suppression of LINE-1 retrotransposition by members of the APOBEC3 family. HeLa non-silencing control and MOV10 KD cells were co-transfected with pJM101/L1.3 (pLINE-1) as described in chapter 3 Figure 3.10A together with either pCMV4-A3A-HA (pA3A), pCMV4-A3B-HA (pA3B), pCMV4-A3G-HA (pA3G) or the pCMV4-GFP-HA (pGFP) control (1 μg). The retrotransposition frequency was determined by G418 selection of the cultures as described in chapter 3 Figure 3.9. **(B) MOV10 is not required for A3G antiviral activity.** HeLa non-silencing control and MOV10 KD cells were co-transfected with pHIV-1_{IIIB}/ΔVif (0.5 μg) and either pA3G or the pGFP control (0.5 μg). Equal amounts of virus normalised by the $p24^{Gag}$ concentration was added to TZM-bl cells to measure the effect on virion infectivity as described in chapter 3 Figure 3.1B. For panel (A) results are normalised to the non-silencing control, which is set at 100%. Values are the mean ± SD of 3 independent experiments.

5.9 Discussion

Although MOV10 overexpression restricts the replication of a diverse panel of retroviruses and retrotransposons, this chapter investigates this anti-retroelement activity in the context of natural levels of MOV10. Several groups have now tested the effect of depleting endogenous MOV10 on the production and infectivity of HIV-1 virions, and contrasting conclusions have been reported whereby Furtak et al detected an approximately 2-fold decrease in virion infectivity, Wang et al observed an approximately 2.5-fold increase in infectivity and Burdick et al reported no difference in infectivity, although a 2-fold decrease in virus production was detected (Burdick et al., 2010; Furtak et al., 2010; Wang et al., 2010). Furthermore, Wang et al also reported an increase in HIV-1 spreading replication with silencing of endogenous MOV10 (Wang et al., 2010). Here this analysis was extended beyond HIV-1 to include SIVmac, MLV and M-PMV, and results from multiple experiments demonstrate no statistically significant effect of silencing endogenous MOV10 on the production, infectivity or replication of HIV-1 (Figures 5.1, 5.3 and 5.4), as well as the production of infectious SIVmac, MLV or M-PMV particles (Figure 5.5). A second unrelated MOV10-specific shRNA produced comparable results (data not shown), although further experiments were not performed with this construct due to the MOV10 KD being less stable in cell lines produced with this shRNA vector.

Several cellular restriction factors are antagonised by the primate lentivirus Vif, Vpu, Vpx, Nef and Env proteins, targeting them for degradation and hence resulting in the counteraction of their antiviral activity (chapter 1 sections 1.8.2.5, 1.8.2.6 and 1.8.2.8). However, MOV10 expression was unaffected by spreading HIV-1 replication confirming that endogenous MOV10 is not degraded by HIV-1 encoded proteins (Figure 5.6). However, this does not rule out a mechanism by which exogenous retroviruses may counteract MOV10 in a degradation-independent manner. Conversely, Wang et al showed HIV-1 replication to decrease the abundance of endogenous MOV10 protein in cell lines and also PBMCs, and this reduction was observed as early as 36hrs post-infection, although MOV10 protein stability and transcript levels were unaffected (Wang et al., 2010). Alternatively, the type I IFN response is activated early during infection and induces the expression of antiviral proteins as well as MOV10 at the mRNA level; therefore, it was hypothesised that during physiological infection endogenous MOV10 protein expression may be induced to levels sufficient for the

restriction of exogenous retroviruses. However, results confirmed no effect of IFNα treatment on MOV10 protein expression in cell lines or HIV-1 primary cell targets (Figures 5.7 and 5.8). Therefore, natural levels of MOV10 do not control the replication of exogenous retroviruses.

It may be argued that residual levels of MOV10 may account for the lack of an effect of endogenous MOV10 KD on exogenous retroviruses, however, notably, silencing of endogenous MOV10 significantly enhances the retrotransposition of LTR and non-LTR endogenous retroelements (Figure 5.9). Furthermore, expression of a silencing resistant version of MOV10 in MOV10 KD cells restores control of LINE-1 retrotransposition, confirming the specificity of this observation (Figure 5.10). Lu et al also recently described ectopically overexpressed MOV10 to suppress IAP retrotransposition in a dose-dependent manner, however; in contrast, they reported a 2-fold decrease in IAP retrotransposition with MOV10 KD (Lu et al., 2012).

To further delve into the molecular mechanism/s by which endogenous MOV10 may control retrotransposition, the cellular associations of MOV10 were considered (chapter 1 section 1.13.1). MOV10 interacts with members of the AGO protein family, which are core components of the RISC involved in miRNA and siRNA-mediated post-transcriptional RNA silencing pathways, and has also been reported to be necessary for miRNA-mediated cleavage of a target reporter mRNA (Meister et al., 2005). Interestingly, LINE-1 encoded endo-siRNAs have been reported to suppress LINE-1 retrotransposition by an RNAi mechanism (Yang and Kazazian, 2006). However, the results presented here confirm that MOV10 is dispensable for endogenous let-7 miRNA-mediated translation repression and slicer activity, at least in HeLa cells (Figures 5.11 and 5.12). It may be that MOV10 interacts with only specific miRNAs, not including let-7; however, the abundance and highly conserved nature of the let-7 miRNA family as well as their multifunctional role in development, cell cycling, proliferation, apoptosis as well as terminal differentiation, makes this seem less likely.

MOV10 interacts and co-localises with post-transcriptional RNA silencing machinery and the APOBEC3 proteins in cytoplasmic P bodies (Meister et al., 2005; Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). Several reports have implicated

APOBEC3 proteins and post-transcriptional RNA silencing pathways in the negative regulation of endogenous retroelements (chapter 1 sections 1.8.2.9, 1.8.2.10 and 1.12.5) and, furthermore, studies have proposed both stimulatory and inhibitory roles for other factors that localise to P bodies in the life cycle of ERVs and retrotransposons as well (chapter 1 section 1.10.1). Lu et al reported silencing of the P body components DDX6 and eIF4E-T to enhance IAP expression, retrotransposition, protein levels and also the accumulation of reverse transcripts (Lu et al., 2011). On the other hand, yeast Ty1 and Ty3 retrotransposons depend on P bodies and localising factors for VLP assembly and retrotransposition (Beliakova-Bethell et al., 2006; Larsen et al., 2008). Interestingly, structure-function studies in chapter 4 revealed that MOV10 mutants demonstrating partial loss of anti-LINE-1 function did not localise to P bodies and even disrupted P bodies (chapter 4 sections 4.6 and 4.7) implying that P bodies may be important for MOV10-mediated suppression of LINE-1 retrotransposition. Considering this it was hypothesised that depletion of endogenous MOV10 may enhance retrotransposition by modulating the size or number of P bodies, however, MOV10 KD had no effect on P bodies disproving this hypothesis (Figure 5.13). Consistently, Lu et al reported MOV10 overexpression to have no effect on the size or number of P bodies as well and, secondly, also proposed that ectopically overexpressed MOV10 was able to effectively suppress IAP retrotransposition in the absence of P bodies (Lu et al., 2012). These results confirm that MOV10 is not an essential P body component and that variations in retrotransposition with MOV10 overexpression or KD are not a consequence of visible modulations in P bodies. Furthermore, the report from Lu et al implies that P bodies may be dispensable for MOV10-mediated control of retrotransposition, however, it will be important to test whether endogenous levels of MOV10 effectively control retrotransposition in the absence of P bodies, as these cytoplasmic foci may essentially function as sites where high levels of endogenous MOV10 may aggregate permitting an amplified response against endogenous retroelements.

Finally, particular interest has been paid to the interaction of MOV10 with antiviral A3F and A3G proteins as Gallois-Montbrun et al initially performed these studies to identify cellular factors that could potentially regulate the editing activity of these enzymes, which presumably left astray would prove detrimental to the host (Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). However, the interaction between MOV10 and APOBEC3 proteins appears not to hold any functional relevance for APOBEC3

anti-retroelement activity as ectopically overexpressed A3A, A3B and A3G suppress LINE-1 retrotransposition as effectively in the presence or absence of endogenous MOV10 (Figure 5.14A). Similarly, ectopically overexpressed A3G inhibits HIV-1 infectivity effectively in MOV10 KD cells (Figure 5.14B). Burdick et al similarly reported the HIV-1 inhibitory activities of A3G and MOV10 to be additive (Burdick et al., 2010). Therefore, MOV10 does not regulate at least the anti-retroelement function of APOBEC3 proteins, and the functional relevance of this association remains to be established.

CHAPTER 6
DISCUSSION AND FUTURE WORK

6.1 Background, findings and outstanding questions

Exogenous and endogenous retroelements are defined by their obligate requirement to reverse transcribe their RNA genome into a DNA copy that is permanently integrated into the host chromosomal DNA, permitting both persistence and transduction into the germ line for vertical transmission. Retroviruses are the causative agents for numerous oncogenic diseases and pathogenic immunodeficiencies, a prime example of which is HIV-1 that is responsible for the global AIDS pandemic. Furthermore, human LINE-1 and Alu somatic retrotransposon insertional events have been associated with several cancers and also implicated in non-hereditary neurobiological disease and diversity [reviewed in (Hancks and Kazazian, 2012)](Coufal et al., 2009; Baillie et al., 2011). Epigenetic regulatory mechanisms and cellular restriction factors such as the APOBEC3 proteins, tetherin, SAMHD1 and potentially TREX1 provide an innate line of defence against the varied cytopathic and mutagenic effects of such genetic parasites (chapter 1 section 1.8). Identification of the full panel of these cellular factors is essential to better understand host-pathogen relationships and, moreover, inform novel therapies.

The findings presented in this thesis focus on the human putative RNA helicase MOV10 and its potential capacity to be a novel cofactor or restriction factor of retroviruses and retrotransposons. The basis of this study originated from the knowledge of multiple reports, primarily including the cellular associations of MOV10 with APOBEC3 proteins, post-transcriptional RNA silencing pathways as well as P bodies and SGs (Meister et al., 2005; Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008) (chapter 1 section 1.13.1). This was further instigated by studies highlighting the crucial role of MOV10 homologs in the suppression of ERVs and retrotransposons in *Drosophila melanogaster* and mice (Frost et al., 2010; Olivieri et al., 2010; Zheng et al., 2010) (chapter 1 section 1.13.2). Demonstrating evolutionary conservation, the results presented in this thesis illustrate that human MOV10 may represent a novel restriction factor of highly active LINE-1 and Alu retrotransposons (chapter 3 section 3.8 and chapter 5 section 5.6). In contrast experiments with exogenous retroviruses revealed

that although ectopically overexpressed MOV10 holds the potential to restrict the replication of viruses belonging to distinct retrovirus genera, natural levels of MOV10 are unable to do so (chapter 3 sections 3.4, 3.6, 3.7 and chapter 5 sections 5.2 and 5.3). Overexpression studies reveal that the inhibitory effect of MOV10 may target multiple stages in the life cycle of retroelements, including reverse transcription (chapter 4 section 4.5), however the molecular mechanism by which MOV10 mediates these effects remains unclear. Nevertheless, MOV10 is dispensable for APOBEC3 protein-mediated restriction of LINE-1 and HIV-1 replication, miRNA-mediated post-transcriptional RNA silencing pathways and also the assembly of P bodies, suggesting that the molecular mechanism by which MOV10 targets retroelements is likely isolated from these known cellular interactions and associations (chapter 5 sections 5.7 and 5.8). Furthermore, highly conserved residues within the MOV10 Walker A and Walker B putative helicase motifs play a necessary, although not sufficient, role in MOV10 anti-retroelement activity (chapter 4 section 4.7).

Taking these results into account, the predominate focus of future work should involve further dissection of remaining MOV10 cellular associations and, subsequently, the identification of a cellular function for MOV10 that may ultimately assist in enlightening the molecular mechanism by which this protein restricts such divergent retroelements. Secondly, overexpression experiments with HIV-1 that have since been confirmed by Lu et al for IAP (Lu et al., 2012) strongly insinuate that MOV10 may target the process of reverse transcription. Therefore, it would be important to, firstly, confirm these findings with endogenous levels of MOV10 and, secondly, identify the mechanism by which MOV10 impairs reverse transcription. Finally, continual detailed structure-function studies would be required to discover residues within MOV10 sufficient for its restrictive properties, also addressing the question of the contribution of MOV10 putative RNA helicase activity. The findings presented in this thesis and future work will be discussed in this chapter in greater detail.

6.2 Ectopically overexpressed MOV10 and retroelements

Overexpression studies are one mechanism by which insights into the potential role of an endogenous protein may be discovered. The results in chapter 3 reveal that MOV10 overexpression decreases the cellular Gag expression of exogenous retroviruses, which may contribute to the reduction in virion production (chapter 3 sections 3.4, 3.6 and

3.7). Pulse-chase analysis will determine whether this reduction in cellular Gag abundance is owed to decreased Gag protein stability or possibly less Gag translation. For the latter, it would also be important to measure viral RNA levels to differentiate between transcriptional or post-transcriptional effects of MOV10 overexpression on Gag translation. MOV10 associates with the AGO proteins, which are central components of the RISC that performs miRNA or siRNA–mediated post-transcriptional RNA silencing. MOV10 depletion studies have suggested that MOV10 may be important for AGO2-mediated slicer activity (Meister et al., 2005). Furthermore, two studies have identified MOV10 as a key regulator of protein translation in rat synapses implicating MOV10 in synaptic plasticity and long-term memory formation (Banerjee et al., 2009; Jarome et al., 2011). Therefore, considering these reports and the colocalisation of ectopically overexpressed MOV10 and HIV-1 Gag in cytoplasmic aggregations closely resembling SGs (chapter 4 section 4.2), it may be that MOV10 controls retroviral Gag translation by sequestering viral RNA in cytoplasmic microdomains and mediating degradation of these transcripts through post-transcriptional RNA silencing pathways. It would be interesting to determine the nature of these cytoplasmic granules and, secondly, determine whether viral RNA colocalises with MOV10 and Gag in these SG-like aggregates. Interestingly, both Gag and genomic RNA can be detected in SGs under certain experimental conditions (unpublished data, Dr. Nathan Sherer).

Conversely, the results presented in chapter 5 demonstrate that MOV10 is dispensable for miRNA-mediated translation repression and cleavage of a reporter mRNA in HeLa cells, arguing against the involvement of such a pathway in the potential regulation of Gag translation (chapter 5 section 5.7). Consistently, ectopically overexpressed MOV10 decreases cellular Gag expression and HIV-1 VLP production from a codon-optimised construct (chapter 4 section 4.3) implying that the MOV10 mechanism of action is independent of sequence-specific determinants within viral transcripts. Therefore, the broad RNA binding capacity of MOV10 may be important for the mechanism by which MOV10 decreases the cellular Gag abundance and production of retroviral particles (Castello et al., 2012). Moreover, MOV10 interacts with a panel of other RBPs with potential roles in translation regulation, as well as the Y RNA-binding Ro protein, which associates with non-coding RNAs, and the RNA helicase Upf1, which mediates mRNA decay (Miki et al., 2011; Sim et al., 2012). These cellular

associations may be significant for the mechanism by which MOV10 decreases cellular Gag expression and virion production and, therefore, future experiments should focus on elucidating the functional relevance of these interactions.

Additionally, at higher MOV10 expression levels Gag processing is also impacted (chapter 3 section 3.4). This may be attributed to reduced intracellular Gag abundance and, therefore, less Gag plasma membrane targeting for assembly. Alternatively, higher concentrations of MOV10 in the cell may be directly detrimental for virion maturation. Supporting the latter claim, electron microscopy (EM) studies from the group of Dr. Vineet KewalRamani detected immature HIV-1 particles budding through the cell plasma membrane with MOV10 overexpression (Retroviruses Meeting, 2010, Cold Spring Harbor Laboratory). It is not understood how ectopically overexpressed MOV10 may directly impair Gag processing, however, this effect seems to be dependent on an increase in MOV10 protein levels above a certain threshold, such as that observed for wild-type HIV-1 experiments in 293T cells (chapter 3 section 3.4).

MOV10 overexpression also produces significantly less infectious HIV-1 virus, and similarly reduces the production of infectious HIV-2, SIVmac, MLV and M-PMV particles (chapter 3 sections 3.4, 3.6 and 3.7). Furthermore, ectopically overexpressed MOV10 also potently suppresses the retrotransposition of the mouse LTR-containing ERV IAP, and human non-LTR LINE-1 and Alu retrotransposons. As MOV10 overexpression decreases the cellular Gag abundance of exogenous retroviruses, it would be interesting to assess whether ectopically overexpressed MOV10 has similar effects on IAP Gag expression and the abundance of LINE-1 proteins as well. Lu et al, however, recently demonstrated MOV10 overexpression to have no effect on IAP Gag levels (Lu et al., 2012).

Interestingly, the highly conserved lysine, aspartic acid and glutamic acid residues in the Walker A and Walker B putative helicase motifs of MOV10 are necessary for its inhibitory activity against both HIV-1 and LINE-1, and the less well conserved glycine amino acid in the Walker B motif is dispensable for MOV10 anti-retroelement activity (chapter 4 section 4.7). However, K530A and DE645AA are only partial loss of function MOV10 mutants suggesting that other residues essential for MOV10 anti-retroelement activity remain to be identified. On the other hand, as either Walker A or

Walker B putative helicase sequences remain intact in the DE645AA or K530A mutants, respectively, it may be that each motif alone is able to accomplish sufficient ATP binding and hydrolysis so that MOV10 is still able to somewhat restrict retroelements. For this reason a single MOV10 putative helicase mutant was also generated in which all three conserved lysine, aspartic acid and glutamic acid residues were substituted with alanine. Although the molecular weight and also expression of this protein was marginally lower than that for wild-type MOV10, this double Walker A and Walker B helicase mutant displayed a complete loss of antiviral function, implying that these conserved residues alone may be sufficient for MOV10 anti-retroelement activity (data not shown). MOV10 also contains three other putative helicase motifs involved in ATP binding and hydrolysis activity, and it would be worth similarly substituting conserved residues within these motifs to determine their relative contribution to MOV10 inhibitory activity.

Abudu et al identified MOV10 residues 99-949 as the minimal antiviral region implicating residues within the N-terminal and C-terminal putative helicase motif as being necessary for the restriction of HIV-1 by MOV10 (Abudu et al., 2012); however, these experiments were conducted through MOV10 N-terminal and C-terminal truncations and considering the sensitivity of MOV10 to such deletions, as reflected by alterations in MOV10 protein expression and subcellular localisation relative to the wild type protein (chapter 4 section 4.6), this method of identifying residues critical for MOV10 anti-retroelement activity seems questionable. Interestingly, murine MOV10 and human MOV10 are over 90% identical at the protein level, however, murine MOV10 is marginally less active against HIV-1, and inhibits MLV with considerably less efficiency than human MOV10 (Wang et al., 2010). Such comparative analysis of MOV10 from various species may assist in narrowing down residues important for human MOV10 inhibitory activity. Importantly, the helicase status of MOV10 has not yet been confirmed; therefore, prior to attributing its function to putative RNA helicase activity, it would be necessary to test MOV10 in a helicase assay. A thin-layer chromatography-based ATP hydrolysis assay using purified protein and radioactively labelled ATP is a semi-quantitative method that could be performed to determine whether MOV10 is an active RNA helicase.

6.3 Endogenous MOV10 and retroelements

Although ectopically overexpressed MOV10 restricts a diverse panel of exogenous and endogenous retroelements, the physiological relevance of these findings for exogenous retroviruses remains uncertain as depletion of endogenous MOV10 by RNAi has no obvious effect on the production, infectivity or replication of HIV-1, or the production of infectious SIVmac, MLV or M-PMV particles (chapter 5 sections 5.2 and 5.3). Yu et al similarly reported silencing of endogenous MOV10 to have no effect on foamy virus replication, which belongs to the distantly related spumavirus subfamily of retroviruses (Yu et al., 2011). For these experiments MOV10 is undetectable at the protein level by immunoblotting and it may be argued that residual levels of undetectable MOV10 may remain functional; however, IAP, LINE-1 and Alu retrotransposition is significantly enhanced with a similar level of depletion of endogenous MOV10.

Exogenous retroviruses, such as the primate lentiviruses, encode several examples of proteins able to degrade host cell restriction factors such as the APOBEC3 proteins, tetherin and SAMHD1 (chapter 1 section 1.8.2). However, spreading HIV-1 replication has no effect on the abundance of endogenous MOV10 protein confirming that a viral protein does not similarly degrade MOV10 to counteract its antiviral activity (chapter 5 section 5.4). Whether a viral protein is able to antagonise MOV10 in a degradation-independent manner remains to be established. Interestingly, MOV10 overexpression decreases the production of HIV-1 VLPs with moderately higher efficacy relative to wild-type virions (chapter 3 section 3.3) and, furthermore, although not statistically significant, the production of infectious HIV-1 vectors appears to be slightly enhanced with MOV10 KD as well (chapter 5 section 5.3). HIV-1 VLPs and vectors are produced from constructs lacking the majority of viral accessory proteins, therefore, it is tempting to speculate that a viral protein absent in the GPV-RRE (chapter 3 section 3.2) or p8.91 (chapter 3 section 3.6) constructs may be able to counteract endogenous MOV10 in the context of the wild-type virus. Supporting this speculation, SIVmac vectors are produced with the pSIV3+ packaging plasmid, which encodes the majority of viral accessory and regulatory proteins (chapter 3 section 3.6), and in this instance unlike for HIV-1 vectors, there is no hint of an increase in production of infectious SIVmac particles with MOV10 KD (chapter 5 section 5.3). Further experiments with lentiviral packaging plasmids harbouring varying deletions in viral accessory proteins will be required to ascertain the reality of this theory.

Alternatively, the antiviral effect of MOV10 overexpression may be recapitulated through stimulation of MOV10 protein expression, for example, by cytokines. The type I IFN response is triggered early during infection and IFNα stimulates the expression of a number of known antiviral proteins. MOV10 protein expression, however, is not induced by IFNα treatment in cell lines or primary CD4+ T cells and MDMs, the natural targets for primate lentiviral infection (chapter 5 section 5.5). Furthermore, IFNα treatment does not alter the subcellular distribution of MOV10 either (chapter 5 section 5.5). IFNβ is also a member of the type I IFN family and the effect of this cytokine on MOV10 protein abundance remains to be determined. Furthermore, HIV-1 infection is associated with the elicitation of a cytokine storm whereby levels of proinflammatory cytokines such as tumour necrosis factor alpha (TNFα) and interleukin-15 (IL-15) are rapidly elevated, along with a slower increase in the levels of interleukin-6 (IL-6) and IFNγ (Stacey et al., 2009). Further experiments should focus on assessing the effects of these cytokines on MOV10 protein expression.

Therefore, endogenous retroelements are sensitive to suppression by natural levels of MOV10; however, the control of exogenous retroviruses may be dependent on a specific threshold of endogenous MOV10 that may be attained by cytokine stimulation during infection. Furthermore, this requirement for higher levels of MOV10 may be attributed to a potential degradation-independent counteraction of MOV10 by a viral accessory protein. Hence, MOV10 may be a component of a pathway or multiple pathways that exogenous retroviruses encounter during physiological infection and future experiments should aim to test these hypotheses.

6.4 MOV10 targets in the retroelement life cycle

Retroviruses package their dimeric genome through interactions between the NC domain of Gag and the Psi stem-loop structure, and this binding is thought to occur in the cytoplasm (chapter 1 section 1.5.9.1). Ectopically overexpressed MOV10 decreases the packaging of HIV-1 genomic RNA into nascent virions, which likely contributes wholly or partially to the infectivity defect in target cells (chapter 4 section 4.4). Ectopically overexpressed MOV10 also decreases HIV-1 cellular Gag abundance and Gag processing (chapter 3 section 3.4). As unspliced viral transcripts serve as both the viral genome and mRNA for translation of Gag and Gag-Pro-Pol precursor proteins,

transcriptional or post-transcriptional targeting of unspliced viral transcripts may decrease viral RNA abundance or availability, and also decrease the expression of viral structural (Gag) and enzymatic proteins (PR, RT and IN). Therefore, a potential target for MOV10 in the HIV-1 life cycle may be unspliced viral transcripts and future studies should focus on testing this hypothesis. Supporting this notion, Burdick et al revealed that HIV-1 viral RNA co-immunoprecipitates with ectopically overexpressed MOV10 (Burdick et al., 2010). Furthermore, Wang et al demonstrated MOV10 overexpression to have no effect on the abundance of unspliced 9kb viral transcripts or spliced mRNAs (Wang et al., 2010) providing precedence for a potential post-transcriptional mechanism for targeting of unspliced viral transcripts. Considering the capacity of MOV10 to bind a broad range of RNAs (Castello et al., 2012) and the fact that MOV10 overexpression decreases the cellular Gag abundance of HIV-2 and M-PMV as well (chapter 3 sections 3.6 and 3.7), targeting of unspliced viral transcripts may be a common mechanism by which MOV10 inhibits exogenous retroviruses.

As an alternative more direct mechanism, MOV10 is packaged into budding HIV-1 virions through an RNA-dependent interaction with the NC region of Gag (Chertova et al., 2006; Wang et al., 2010; Abudu et al., 2012) and, therefore, MOV10 overexpression may physically impede the association of viral RNA with NC for packaging. The nature of the RNA species necessary for MOV10 packaging into virions is unclear although MOV10 is incorporated into virions in the absence of viral RNA, albeit with moderately less efficiency (Burdick et al., 2010). On the contrary, overexpression of MOV10 has been reported to have no effect on IAP Gag levels or RNA incorporation into VLPs (Lu et al., 2012) implying that there may be some intrinsic differences between the regulation of exogenous retroviruses and ERVs, or alternatively IAP assembly at the ER as opposed to the plasma membrane may be accountable for these variable observations.

HIV-1 virions produced from cells overexpressing MOV10 are also defective in the accumulation of reverse transcription products in target cells (chapter 4 section 4.5), which is not attributed to a defect in target cell attachment or entry (Furtak et al., 2010; Wang et al., 2010). Although this may be an indirect effect of decreased genome incorporation into virions, the impact on viral cDNA synthesis is more potent that the reduction in viral RNA packaging suggesting that ectopically overexpressed MOV10

may directly impair reverse transcription as well. Interestingly, in the event that MOV10 targets unspliced viral transcripts the abundance of the RT enzyme would also be reduced, which would account for the defect in reverse transcription. Future experiments should aim to analyse the effect of MOV10 overexpression on the abundance of viral PR, RT and IN enzymes.

Alternatively, as endogenous MOV10 is also packaged into HIV-1 virions (Chertova et al., 2006) it may directly interfere with the process of reverse transcription. However, the functional relevance of MOV10 incorporation into virions in terms of its antiviral activity remains unclear. Structure-function studies from the group of Dr. Vineet KewalRamani revealed that the antiviral N-terminal domain of MOV10 is not packaged into HIV-1 virions, and in contrast the C-terminal loss of function MOV10 putative helicase domain is efficiently incorporated into nascent particles (Retroviruses Meeting, 2010, Cold Spring Harbor Laboratory). Although these data negate a correlation between MOV10 packaging and antiviral activity, conclusions cannot be drawn from such experiments owing to the variable expression and subcellular localisation of these truncated MOV10 mutants compared to the full-length protein (chapter 4 section 4.6). Comparisons between the packaging efficiency and antiviral activity of MOV10 putative helicase mutants K530A, DE645AA and G648A may provide a better system for addressing this question.

Analogous to the observations with HIV-1, Lu et al reported MOV10 overexpression to decrease the accumulation of IAP reverse transcription products and, furthermore, MOV10 was also packaged into IAP VLPs (Lu et al., 2012). LINE-1 and Alu retrotransposons couple reverse transcription and integration through a process called TPRT that takes place in the nucleus (chapter 1 section 1.7.1). The effect of MOV10 on TPRT, if any, remains to be elucidated. It is difficult to comprehend how MOV10 may directly interfere with TPRT considering that the subcellular localisation of MOV10 is cytoplasmic (chapter 4 section 4.6). However, analysis of MOV10 steady-state distribution by immunofluorescence may overlook any potential shuttling of MOV10 into the nucleus. Additionally, despite the positive correlation between nuclear localisation of APOBEC3 proteins and suppression of LINE-1 retrotransposition, Pak et al recently demonstrated that targeting of otherwise nuclear A3B to the cytoplasm by mutating four residues at the N-terminus of the protein retained the capacity of A3B to

suppress LINE-1 replication (Pak et al., 2011). Extrapolating from observations with HIV-1, it would be important to explore the effect of MOV10 overexpression on the abundance and localisation of LINE-1 RNA and proteins, as ectopically overexpressed MOV10 may suppress retrotransposition by similarly targeting retrotransposon transcripts and, therefore, impacting the expression of LINE-1 proteins and downstream processes such as TPRT in this manner.

Therefore, studies with wild-type HIV-1 have established that MOV10 overexpression affects multiple stages of the HIV-1 life cycle including cellular Gag expression, Gag processing, viral RNA packaging and reverse transcription (chapter 3 section 3.4 and chapter 4 sections 4.4 and 4.5). Although the cellular Gag abundance of HIV-2 and M-PMV is also decreased (chapter 3, sections 3.6 and 3.7), a thorough investigation is necessary to determine whether these observations are true for all retroviruses and also retrotransposons tested. Furthermore, it remains to be seen whether MOV10 possesses the capacity to target each of these various stages individually, or whether it mediates its inhibitory activity by targeting the genomic RNA of these retroelements resulting in the establishment of the multiple downstream defects. The latter hypothesis is further supported by the ability of MOV10 to associate with a broad range of RNAs with limited specificity (Castello et al., 2012), as well as unspliced HIV-1 transcripts (Burdick et al., 2010). Finally, these experiments are performed with ectopically overexpressed MOV10 and, therefore, eventually it would be important to assess the capacity of natural levels of MOV10 to target these stages of the retroelement life cycle.

6.5 Importance of cellular associations for MOV10 mechanism of action

The list of MOV10 cellular associations is extensive most likely reflecting the ability of MOV10 to associate with a broad range of RNAs (Castello et al., 2012). Consistently, MOV10 interacts with several RBPs and localises to cytoplasmic P bodies and SGs, which are sites for mRNA storage and degradation (chapter 1 section 1.13.1). The cellular function/s of human MOV10 remains unknown, however, studies in rat hippocampal and amygdala cells have revealed an essential role for MOV10 in translational control at synapses (Banerjee et al., 2009; Jarome et al., 2011). The identification of functionally relevant MOV10 cellular associations is essential to discover the cellular function/s of human MOV10 and, moreover, establish the molecular mechanism by which MOV10 restricts retroelements.

Evidence from several studies now suggests that MOV10 likely exists in an RNP complex in association with antiviral APOBEC3 and AGO proteins, and these RNP complexes localise to P bodies and SGs (Meister et al., 2005; Gallois-Montbrun et al., 2007; Gallois-Montbrun et al., 2008). Consistent with its interaction with AGO proteins, MOV10 has been proposed to be necessary for miRNA-mediated cleavage of a reporter mRNA. This suggests that a cellular function of MOV10 may be miRNA-mediated post-transcriptional RNA silencing, which agrees with the reports confirming a role for MOV10 in translation regulation in the rat brain (Banerjee et al., 2009; Jarome et al., 2011). However the results presented in chapter 5 demonstrate that MOV10 is dispensable for both endogenous let-7 miRNA-mediated translation repression and slicer activity in HeLa cells, at least in the context of reporter constructs (chapter 5 section 5.7). It would be important to repeat these experiments in other cell types to ensure that this effect is not HeLa cell-specific and, secondly, despite the multifunctional role of endogenous let-7 miRNAs and their high abundance, assays employing other endogenous miRNAs should also be tested. Nevertheless, these data imply that MOV10 may not restrict the replication of retroelements through miRNA-mediated translation repression or RNA cleavage pathways. To verify this further, the inhibitory activity of MOV10 may be tested in DICER-1 depleted cells. .

A previous report has proposed that a cellular function of A3G may be the derepression of miRNA-mediated translation repression (Huang et al., 2007), and more recently this group has suggested that A3G mediates these effects by interfering with the interaction between MOV10 and AGO2, ultimately blocking maturation of the RISC (Liu et al., 2012). However, Phalora et al confirmed no effect of APOBEC3 proteins on miRNA function (Phalora et al., 2012) and, similarly, the findings presented here do not identify a role for MOV10 in miRNA-mediated translation repression either. Based on these data and the independent anti-retroelement activities of MOV10 and APOBEC3 proteins (chapter 5 section 5.8), the role of the MOV10, APOBEC3 and AGO protein RNP complex remains unclear, and does not seem to be functionally relevant at least in the context of MOV10 inhibitory activity.

MOV10 localises in P bodies and redistributes to SGs under conditions of cellular stress (Gallois-Montbrun et al., 2007). P bodies and localising factors have been implicated in the negative and positive regulation of exogenous and endogenous retroelements

(chapter 1 section 1.10.1). However, Phalora et al recently confirmed no effect of depleting P bodies through knockdown of DDX6 and Lsm1 on HIV-1 replication (Phalora et al., 2012). In contrast, Lu et al reported knockdown of DDX6 and eIF4E-T to enhance IAP retrotransposition (Lu et al., 2011), implying that although P bodies do not regulate HIV-1 replication, they may still play a role in the life cycle of endogenous retroelements. MOV10 is not an essential P body factor as depletion (chapter 5 section 5.7) or overexpression of MOV10 (Lu et al., 2012) has no affect on the size or number of P bodies. Furthermore, ectopically overexpressed MOV10 inhibits IAP retrotransposition effectively in P body depleted cells (Lu et al., 2012) suggesting that P bodies may be dispensable for MOV10-mediated restriction of endogenous retroelements. It would be important to determine whether P bodies are similarly dispensable for the suppression of retrotransposition by natural levels of MOV10 as these cytoplasmic foci may reflect sites for endogenous MOV10 aggregation, which may permit a more effective response again endogenous retroelements. In contrast, this aggregation may not be necessary in the context of MOV10 overexpression due to the generally increased concentration of MOV10 in cells.

Alternatively, considering that ectopically overexpressed MOV10 and HIV-1 Gag co-localise in SG-like cytoplasmic aggregates (chapter 4 section 4.2), and HIV-1 Gag and viral RNA can be detected in SGs under certain experimental conditions as well (unpublished data, Dr. Nathan Sherer), the role of SGs in MOV10 anti-retroelement activity warrants further investigation. HIV-1 Gag and genomic RNA also associate with Staufen1 RNP complexes that are distinct from P bodies and SGs (Abrahamyan et al., 2010), and considering the previously identified interaction of MOV10 with Staufen2 RNP complexes (Miki et al., 2011) it would be worth exploring whether MOV10 also associates with Staufen1 RNP complexes and the relevance, if any, of this potential interaction. Nonetheless, MOV10 interacts with a magnitude of other RBPs such as ZBP1, YB-1, Upf1 and RHA, which have been implicated in cellular processes such as translation control, mRNA degradation and RISC loading (Miki et al., 2011; Sim et al., 2012). It would be necessary to establish the functional relevance of these cellular associations as a way to potentially identify a cellular role for MOV10 and, moreover, isolate the mechanism by which MOV10 is able to restrict a broad panel of retroviruses and retrotransposons.

Furthermore, extrapolating from studies with MOV10 homologs may also provide an insight into the MOV10 mechanism of action. MOV10L1 is the germline-restricted mammalian paralog of MOV10 that interacts with mammalian PIWI proteins and is essential for piRNA-guided suppression of ERVs and retrotransposons through DNA methylation. Therefore, future research should be extended to similarly assess the capacity of MOV10 to control endogenous retroelements at the level of transcription through epigenetic regulatory mechanisms. Interestingly, naturally occurring endo-siRNAs have been proposed to regulate the expression of LINE-1 through DNA methylation of the LINE-1 promoter in human somatic cells and, moreover, breast cancer cells that are associated with increased LINE-1 expression display reduced levels of these endo-siRNAs (Chen et al., 2012). Furthermore, supporting the association of MOV10 with epigenetic regulatory pathways, Messaoudi-Aubert et al revealed MOV10 to interact with components of the Polycomb-repressive complex 1 in human cells, which together with the Polycomb-repressive complex 2 mediates transcriptional repression through the methylation and ubiquitination of nucleosomal histones (Messaoudi-Aubert et al., 2010).

6.6 A physiological role for MOV10: A bigger picture

The human germline and, as discovered more recently, somatic cells are prone to mutagenic effects attributed to retrotransposition events. LINE-1 expression is detectable in a wide range of human tissues (Belancio et al., 2010), and to date approximately 96 single gene diseases have been caused by retrotransposon insertions a huge proportion of which are, unsurprisingly, cancers [reviewed in (Hancks and Kazazian, 2012)]. LINE-1 and Alu activity is elevated in several cancers and this phenomenon correlates with hypomethylation of retrotransposon promoters implying that a breakdown in epigenetic regulatory mechanisms that would normally suppress retrotransposition may facilitate oncogenic insertional mutations [reviewed in (Belancio et al., 2010)](Iskow et al., 2010). Coufal et al revealed LINE-1 expression to be elevated in the human brain relative to other organs (Coufal et al., 2009) and, fascinatingly, Baillie et al identified over 20,000 novel LINE-1 and Alu insertions in the human hippocampus and caudate nucleus (Baillie et al., 2011) highlighting the human brain as a hotspot for somatic retrotransposition. These reports stress the significance of retrotransposition as a potential non-hereditary mechanism contributing to neurological diversity and disease. Nevertheless, Lee et al recently discovered somatic

retrotransposon insertions in epithelial tumours, although not in cancers of the brain (Lee et al., 2012). These data may reflect the presence of regulatory pathways or cellular restriction factors in non-transformable cell types that may be missing or expressed at lower levels in cells susceptible to transformation.

Interestingly, human MOV10 transcripts are expressed at the highest level in the adult CNS including the hippocampus and caudate nucleus (Nagase et al., 2000) and, furthermore, MOV10 protein abundance is also increased in cancer cells compared to normal healthy cells (Nakano et al., 2009). Considering the elevated expression of retrotransposons in the human brain and various cancers, it would be interesting to determine whether the correlative MOV10 expression pattern holds any functional relevance. The results presented in chapter 5 demonstrate that natural levels of MOV10 suppress the retrotransposition of human LINE-1 and Alu retrotransposons (chapter 5 section 5.6), therefore, it may be hypothesised that higher expression levels of MOV10 in the human brain may control the mobilisation of retrotransposons during conditions of deregulation, such as neurogenesis, limiting the likelihood of a retrotransposon insertion with a mutagenic consequence. Alternatively, lower expression levels of MOV10 in other tissues may not suffice in the suppression of retrotransposons increasing the probability of insertional mutagenesis. Therefore, similar to MOV10L1 in the germline, the role of MOV10 may be to suppress the retrotransposition of endogenous retroelements in somatic cells as a mechanism to protect cells exposed to high levels of retrotransposon deregulation from deleterious mutations.

The retrotransposition assay may be employed for preliminary experiments to compare the effects of endogenous MOV10 depletion on LINE-1 and Alu replication in cells from the human brain versus those from tissues expressing lower levels of MOV10. Although a MOV10 knockout mouse has not yet been generated, such mice would be extremely valuable to feasibly test this hypothesis. Interestingly, blood cells are also refractory to transformation by retrotransposon insertions (Lee et al., 2012) and, therefore, it would be worth comparing MOV10 expression levels in cells from the blood, brain and other tissues reported to express lower levels of MOV10 (Nagase et al., 2000).

6.7 Summary

In view of MOV10 cellular associations and homolog functions, the aim of this thesis was to broadly ascertain the role of the human putative RNA helicase MOV10 in the replication of a panel of genetically diverse exogenous and endogenous retroelements, including members from the lentivirus, gammaretrovirus and betaretrovirus subfamilies, as well as an LTR-containing ERV and non-LTR retrotransposons. In summary, the results presented in this thesis demonstrate that ectopically overexpressed MOV10 possesses the capacity to potently restrict all retroviruses and retrotransposons tested. Importantly however, RNAi-mediated depletion of endogenous MOV10 has no effect on the replication of exogenous retroviruses, although specifically enhances the propagation of endogenous retroelements. These results demonstrate that natural levels of MOV10 suppress the retrotransposition of LTR and non-LTR endogenous retroelements. Although endogenous MOV10 protein levels are unaffected by retroviral replication excluding the possibility of a viral protein degrading MOV10 as a counteraction mechanism, the possibility of a degradation-independent mechanism of MOV10 antagonism remains to be investigated. Furthermore, MOV10 protein expression is not induced by IFNα treatment ruling out a scenario in which MOV10 levels may be sufficiently stimulated for the restriction of exogenous retroviruses mimicking the overexpression results. However, a magnitude of other cytokines are triggered during natural infection and the effect of these on MOV10 protein abundance remains to be established.

Ectopically overexpressed MOV10 targets both early and late stages of the HIV-1 life cycle resulting in a decrease in cellular Gag abundance, virion processing and maturation, genomic RNA packaging and accumulation of reverse transcription products. Further studies should extend these findings to other retroviruses and retrotransposons and, moreover, investigate the ability of natural levels of MOV10 to target these multiple stages. It is unclear if ectopically overexpressed MOV10 targets each of these various stages individually or whether these phenotypes are a consequence of MOV10 targeting retroelement transcripts either transcriptionally or post-transcriptionally. Structure-function studies have highlighted conserved residues within the MOV10 Walker A and Walker B putative ATP-binding and hydrolysis motifs as being necessary for MOV10 anti-retroelement activity; although these are not sufficient

for MOV10 inhibitory activity and other key residues will need to be identified through similar experiments. Significantly, these results reveal a potential requirement for MOV10 putative RNA helicase activity for the restriction of retroelements. The molecular mechanism by which MOV10 targets retroviruses and retrotransposons is still a mystery. Focusing on the cellular associations of MOV10 it is now clear that the anti-retroelement activities of MOV10 and members of the APOBEC3 protein family are independent, and that MOV10 is not essential for miRNA-mediated post-transcriptional translation repression or mRNA cleavage pathways in cultured cells. It is important to continue dissecting the cellular associations of MOV10 as a way to potentially identify the cellular role of MOV10 and the mechanism for its inhibitory activity.

In sum, MOV10 possesses the capacity to restrict the replication of a broad range of genetically distinct retroviruses and retrotransposons implying that MOV10 is a component of a cellular pathway or process that retroelements encounter; however, further investigation is necessary to provide a physiological understanding of the relationship between MOV10 and exogenous retroviruses. Significantly, the specificity of natural levels of MOV10 in the suppression of endogenous retroelements highlights MOV10 as a potential novel restriction factor of retrotransposons in somatic cells. This is valuable knowledge with possible therapeutic implications considering that human retrotransposon insertions are now known to be the causative agents of countless single gene diseases including several cancers, haemophilia and cystic fibrosis, and have also been suggested to contribute to non-hereditary neurological diversity and disease.

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PUBLICATION

RETROVIROLOGY

RESEARCH Open Access

Endogenous MOV10 inhibits the retrotransposition of endogenous retroelements but not the replication of exogenous retroviruses

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Abstract

Background: The identification of cellular factors that regulate the replication of exogenous viruses and endogenous mobile elements provides fundamental understanding of host-pathogen relationships. MOV10 is a superfamily 1 putative RNA helicase that controls the replication of several RNA viruses and whose homologs are necessary for the repression of endogenous mobile elements. Here, we employ both ectopic expression and gene knockdown approaches to analyse the role of human MOV10 in the replication of a panel of exogenous retroviruses and endogenous retroelements.

Results: MOV10 overexpression substantially decreased the production of infectious retrovirus particles, as well the propagation of LTR and non-LTR endogenous retroelements. Most significantly, RNAi-mediated silencing of endogenous MOV10 enhanced the replication of both LTR and non-LTR endogenous retroelements, but not the production of infectious retrovirus particles demonstrating that natural levels of MOV10 suppress retrotransposition, but have no impact on infection by exogenous retroviruses. Furthermore, functional studies showed that MOV10 is not necessary for miRNA or siRNA-mediated mRNA silencing.

Conclusions: We have identified novel specificity for human MOV10 in the control of retroelement replication and hypothesise that MOV10 may be a component of a cellular pathway or process that selectively regulates the replication of endogenous retroelements in somatic cells.

Keywords: MOV10, Retrovirus, Retrotransposon, APOBEC3

Background

Exogenous retroviruses and endogenous retroelements replicate in the host by reverse transcribing their RNA genomes into DNA copies that are permanently integrated into the host genome, making them some of the most successful parasites studied. Approximately 45% of the human genome is derived from mobile elements, with active long interspersed nucleotide element-1 (LINE-1), Alu and SINE-R/VNTR/Alu (SVA) retrotransposition events contributing to disease-producing insertional mutations in humans [1-4]. Host cells have evolved multiple transcriptional and post-transcriptional control mechanisms to protect themselves and their genomes from the pathogenic and mutagenic effects of such parasites.

Cellular restriction factors form an effective innate defence against a range of exogenous retroviruses and intracellular retroelements. The human APOBEC3 (*apo*lipoprotein B mRNA-*e*diting enzyme *c*atalytic polypeptide 1-like 3) family of cytidine deaminases are potent intrinsic antiviral factors that restrict a broad range of exogenous retroviruses [5-9] as well as the propagation of numerous endogenous retroelements [6-10]. Similarly, TRIM5α [11], tetherin [12] and SAMHD1 [13,14] are restriction factors that can inhibit the replication of exogenous retroviruses at different steps in the retroviral life cycle [15]. Intriguingly, the cytosolic exonuclease TREX1 metabolises reverse-transcribed DNA derived from endogenous retroelements and, presumably, restricts their retrotransposition [16], yet is a co-factor for human immunodeficiency virus type-1 (HIV-1)

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infection [17] revealing the complexity of host-pathogen interactions.

MOV10 (Moloney leukaemia virus 10) is a superfamily 1 (SF1) putative RNA helicase that acts as a co-factor or inhibitory factor for a number of RNA viruses. MOV10 is required for the replication of human hepatitis delta virus (HDV) [18] but restricts hepatitis C virus (HCV) and vesicular stomatitis virus (VSV) replication [19,20]. The antiviral function of MOV10 is evolutionarily conserved as its ortholog in *Arabidopsis thaliana*, SDE3 (silencing defective protein 3), regulates small-RNA mediated silencing of specific exogenous viruses [21], whereas its ortholog in *Drosophila melanogaster*, Armitage, and its mammalian paralog, MOV10-like-1 (MOV10L1), are necessary for piRNA-mediated repression of endogenous retroelements [22-26]. MOV10 has also been reported to associate with the RNA-induced silencing complex (RISC) that mediates small RNA-mediated RNA silencing [27]. Recently, we and others identified MOV10 as interacting with the antiviral APOBEC3 proteins, APOBEC3G (A3G) and APOBEC3F (A3F), in an RNA-dependent manner [28,29]. Taken together, these observations suggest that human MOV10 may regulate a wide range of RNA viruses and could also control the retrotransposition of endogenous retroelements in mammals.

Supporting the hypothesis that MOV10 is an antiviral factor, several groups have reported that MOV10 overexpression restricts the infectivity of HIV-1 and other retroviruses [30-33], although the proposed mechanisms of action differ. Endogenous MOV10 is packaged into HIV-1 virions produced from infected monocyte-derived macrophages, and recently it was reported that MOV10 packaging requires the nucleocapsid region of Gag [30,32-34]. Crucially, these reports varied substantially in their conclusions regarding the effect of depleting endogenous MOV10 on HIV-1 replication in that they either observed a slight decrease in infectivity [31], a modest increase in infectivity [32], or a small decrease in virus production with no difference in infectivity [30]. These contrasting results have led to confusion over whether MOV10 is a co-factor or an inhibitory factor for HIV-1 replication. Furthermore, the possible role of MOV10 in regulating the replication of endogenous retroelements in mammalian cells awaits examination.

To define MOV10′s capacity to regulate retroelements, we undertook side-by-side comparisons of the effects of MOV10 overexpression and depletion on the replication of a number of exogenous retroviruses and the retrotransposition of endogenous retroelements. Our results indicate that MOV10 overexpression restricts the production of infectious virions for a broad range of exogenous retroviruses and also potently inhibits the mobilisation of endogenous retroelements. Importantly, silencing of endogenous MOV10 has no effect on the replication of exogenous retroviruses though it significantly enhances the transposition of human endogenous retrotransposons and a mouse endogenous retrovirus. Furthermore, we report that MOV10 is not necessary for miRNA or siRNA-mediated RNA silencing in cultured cells.

Results

MOV10 overexpression restricts the production of infectious retrovirus particles

To determine whether the overexpression of MOV10 affects HIV-1 virion production and infectivity, we co-transfected HeLa or 293T cell lines with pHIV-1_{NL4-3} [35] and increasing amounts of pMOV10 or a pluciferase (pLuc) control vector (pT7-MOV10 or pT7-Luc). The virion concentration was determined by $p24^{Gag}$ enzyme-linked immunosorbent assay (ELISA). We observed a consistent dose-dependent decrease in the production of virions from HeLa and 293T cells, whereby at the maximum dose of pMOV10 virus production was reduced by ~70% and ~80%, respectively (Figure 1A). We then tested the infectivity of these virions by adding equal amounts of virus normalised by the $p24^{Gag}$ concentration to the TZM-bl reporter cell line. Overexpression of MOV10 decreased the infectivity of HIV-1 virions substantially in a dose-dependent manner, and at the maximum amount of pMOV10 infectivity was reduced by ~80% for HeLa cells and to undetectable levels for 293T cells (Figure 1B). Cell lysates were analysed by immunoblotting to determine whether MOV10 overexpression affected Gag expression or processing. We quantified all the Gag bands to measure total cellular Gag levels and also determined the percentage of Gag processing (total processed Gag bands divided by total Gag bands). Total cellular Gag levels decreased by ~40% and ~50% in HeLa and 293T cells, respectively, at the maximum pMOV10 amount when compared with the pLuc control (Figure 1A, compare lanes 1 and 7). Furthermore, Gag processing was slightly reduced by ~10% and ~40% in HeLa and 293T cells, respectively (Figure 1A, compare lanes 1 and 7). Therefore, the overexpression of MOV10 decreased the production and infectivity of HIV-1 virions in a dose-dependent manner, and also caused a modest decrease in Gag expression and processing.

We then determined whether MOV10 overexpression also restricts the infectivity of a selection of divergent retroviruses including rhesus macaque-derived simian immunodeficiency virus (SIVmac, a lentivirus), murine leukaemia virus (MLV, a gammaretrovirus) and Mason-Pfizer monkey virus (M-PMV, a betaretrovirus). We produced SIVmac vectors by transfecting 293T cells with an SIVmac Gag-Pol packaging plasmid (pSIV3-RMES4) [36], a GFP-expressing SIVmac vector (pSIV-RMES4) [36] and pVSV-G [37]. As a control, we also tested analogous VSV-G pseudotyped

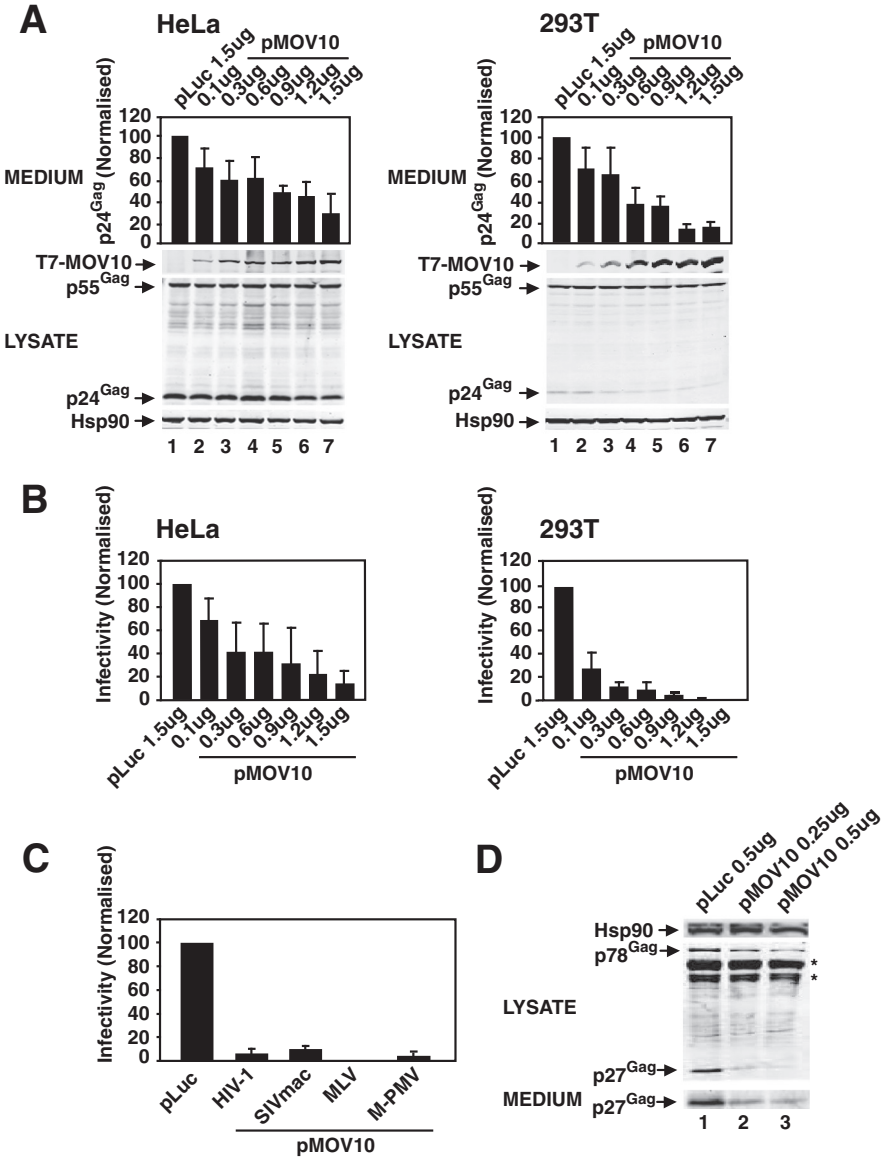

Figure 1 MOV10 overexpression restricts the production of infectious retrovirus particles for a broad range of exogenous retroviruses. **(A)** MOV10 overexpression decreases HIV-1 virus production. HeLa or 293T cells were co- transfected with pHIV-1_{NL4-3} and increasing amounts of pT7-MOV10 as indicated or the pT7-Luc control. Virus concentration in the medium was determined by a $p24^{Gag}$ ELISA. Cell lysates were analysed by quantitative immunoblotting with anti-T7, anti- $p24^{Gag}$ and anti-Hsp90 antibodies. **(B)** Overexpression of MOV10 inhibits the infectivity of HIV-1 virions. The TZM-bl reporter cell line expressing a HIV-1 Tat inducible β-gal reporter gene was infected with equal amounts of virus normalised by the $p24^{Gag}$ concentration from each of the indicated samples. Cells were lysed and β-gal activity was measured to determine virus infectivity. **(C)** MOV10 overexpression inhibits the production of infectious SIVmac, MLV and M-PMV particles. For HIV-1 and SIVmac lentiviral vector production, 293T cells were co-transfected with p8.91, pCSGW and pVSV-G, or pSIV3+, pSIV-RMES4 and pVSV-G, respectively, together with pT7-MOV10 or pT7-Luc. 293T cells were infected with lentiviral particles and infectivity was determined by measuring the percentage of GFP-positive 293T cells by FACS. For MLV and M-PMV virion production, 293T cells were co-transfected with pNCS/FLAG, pMSCV/Tat and pVSV-G, or pMTΔE and pVSV-G, respectively, together with pT7-MOV10 or pT7-Luc. Infectivity was determined using TZM-bl cells. **(D)** Overexpression of MOV10 decreases the production of M-PMV virions. Cell lysates and sucrose cushion purified M-PMV virions were analysed by immunoblotting with anti $p27^{Gag}$ and anti-Hsp90 antibodies (* refers to non-specific bands). For (A), (B) and (C) results are normalised to the pLuc control, which is set at 100%. For (C) a single control bar set at 100% is graphed for simplicity. Values are the mean ± SD of 3 independent experiments.

HIV-1 vectors, produced using the HIV-1 Gag-Pol plasmid p8.91 [38], the HIV-1 GFP-expressing vector pCSGW [39] and pVSV-G. Plasmids for lentiviral vector production were co-transfected with either pMOV10 or the pLuc control. The effect on the production of infectious particles was determined by challenging 293T cells and measuring the

percentage of GFP-positive cells. Similar to the wild-type HIV-1 experiments (Figure 1B), overexpression of MOV10 reduced the production of infectious HIV-1 and SIVmac particles by over 80% relative to the pLuc control (Figure 1C).

To test MLV infectivity, we co-transfected 293T cells with a full-length MLV proviral plasmid (pNCS/FLAG) [40] together with a surrogate MLV genome expressing HIV-1 Tat (pMSCV/Tat) [41] and pVSV-G. To analyse M-PMV infectivity, 293T cells were co-transfected with a M-PMV proviral plasmid in which *env* is replaced with HIV-1 *tat* (pMTΔE) [42] together with pVSV-G. Plasmids for the production of both MLV and M-PMV virions were co-transfected together with either pMOV10 or the pLuc control. The effect on the production of infectious MLV and M-PMV virions was determined by infecting the TZM-bl reporter cells. Overexpression of MOV10 decreased the production of MLV and M-PMV infectious virions by over 80% with respect to the pLuc control (Figure 1C). We also analysed sucrose cushion purified M-PMV virions and cell lysates by immunoblotting to determine the effect of MOV10 overexpression on virus production. Similar to HIV-1 (Figure 1A), we observed a decrease in M-PMV precursor $p78^{Gag}$ and processed $p27^{Gag}$ levels in the cell lysate as well as a decrease in virion production with increasing concentrations of pMOV10 relative to the pLuc control (Figure 1D, compare lane 1 with lanes 2 and 3). These results show that MOV10 overexpression can restrict the production and infectivity of retroviruses from multiple genera.

Overexpression of MOV10 inhibits the retrotransposition of LTR and non-LTR endogenous retroelements

Similar to exogenous retroviruses, endogenous retroelements replicate via an RNA intermediate that is reverse transcribed and integrated into the host genome. Considering the association of MOV10 homologs with the suppression of endogenous mobile elements [22-24], we next assessed whether overexpression of MOV10 inhibits the retrotransposition of some representative endogenous retroelements. We tested the non-LTR autonomous human LINE-1 and its dependent non-autonomous short interspersed nucleotide element (SINE) Alu retrotransposons, both of which reverse transcribe by target-site primed reverse transcription (TPRT) in the nucleus [1]. We also included the mouse intracisternal A-type particle (IAP), which is related to the betaretrovirus family of exogenous retroviruses, though it has a strictly intracellular life cycle [43].

Established cell culture-based retrotransposition assays were used to study these retroelements [44-46]. Briefly, HeLa cells were transfected with expression plasmids for human LINE-1 (pJM101/L1.3) [47], human Alu (pAlu-neo^{Tet}) [48] or mouse IAP (pGL3-IAP92L23neo^{TNF}) [49] all of which contain an antisense neomycin resistance gene cassette (*neo*) in the 3′UTR driven by its own promoter and disrupted by an intron. *Neo* expression occurs only after a full retrotransposition event: specifically, transcription of the retroelement RNA, removal of the intron by splicing, translation of the proteins, reverse transcription and then integration of the cDNA into the host cell genome, allowing for enumeration of retrotransposition by counting G418-resistant colonies. The Alu element is dependent on LINE-1 enzymes for replication; therefore, to measure Alu element retrotransposition, the cells were also co-transfected with a plasmid encoding the LINE-1 ORF2 protein (pCEP-ORF2) [48], which encodes the LINE-1 endonuclease and reverse transcriptase enzymatic activities. Either pMOV10 or the pLuc control was co-transfected to determine the effect of MOV10 overexpression on the replication of these endogenous retroelements. Similar to the observations made with exogenous retroviruses (Figure 1), overexpression of MOV10 decreased human LINE-1, Alu and mouse IAP retrotransposition by over 90% when compared with the pLuc control (Figure 2A).

As a control, HeLa cells were also transfected with a pcDNA3.1 vector that contains a neomycin resistance expression cassette (pcDNA3.1-*neo*) to ensure that MOV10 overexpression did not affect *neo* expression or selection directly. The cultures were G418-selected and the colonies were counted as described for the retrotransposition assays, with similar numbers of colonies seen in the context of MOV10 overexpression as for the pLuc control (Figure 2B). Therefore, MOV10 overexpression inhibits the propagation of multiple endogenous retroelements.

Silencing endogenous MOV10 does not affect the production of infectious retroviral particles

We next determined the effect of depleting endogenous MOV10 on HIV-1 production and infectivity in the context of one full cycle of viral replication. Stable HeLa and 293T non-silencing control and MOV10 knockdown (KD) cell lines were produced by transducing HeLa or 293T cells with lentiviral vectors expressing either a non-silencing control shRNA or a MOV10-specific shRNA, which reduced MOV10 protein steady-state abundance to undetectable levels when compared with the non-silencing control cells (Figure 3A). The depletion of endogenous MOV10 did not affect the growth rate of these cells (data not shown). HeLa or 293T non-silencing control and MOV10 KD cell lines were infected with equal amounts of VSV-G pseudotyped $HIV\text{-}1_{NL4\text{-}3}$ and virion production and infectivity were determined. Depletion of endogenous MOV10 showed no significant effect on the amount (Figure 3A) or infectivity (Figure 3B) of virions produced

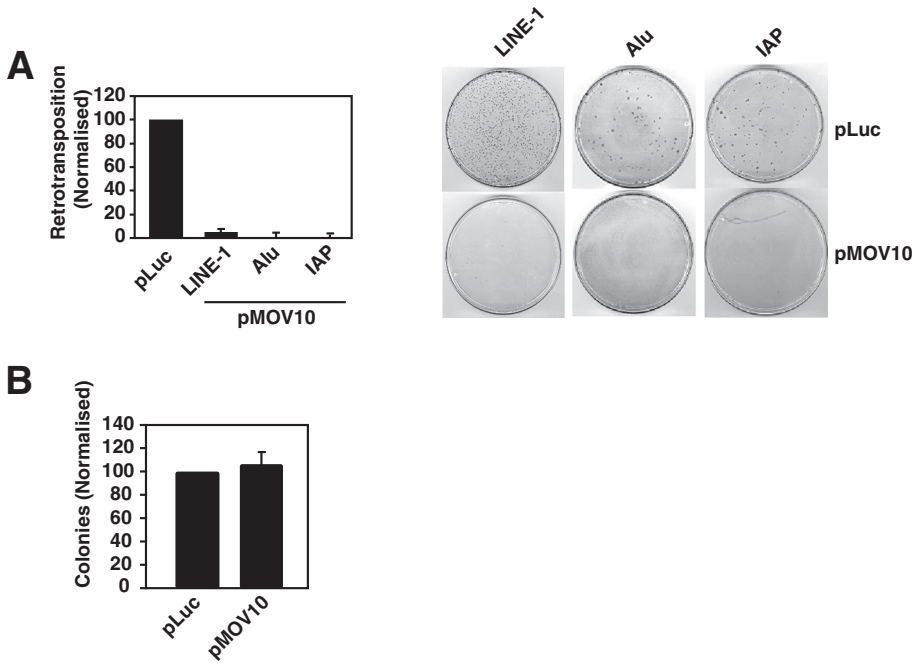

Figure 2 Overexpression of MOV10 suppresses the retrotransposition of both LTR and non-LTR endogenous retroelements. (A) MOV10 overexpression restricts the replication of LINE-1, Alu and IAP. HeLa cells were co-transfected with pLINE-1 (pJM101/L1.3), pAlu (pAlu-neo^{Tet} plus pCEP-ORF2), or pIAP (pGL3- IAP92L23neo^{TNF}) together with pT7-MOV10 or pT7-Luc. Cells were selected with G418 for 12-14 days to measure retrotransposition frequency and then fixed and stained with Giemsa. **(B)** MOV10 overexpression has no affect on *neo* expression or selection. HeLa cells were co-transfected with a pcDNA3.1 control vector containing a neomycin resistance cassette (pcDNA3.1-*neo*) together with pT7-MOV10 or pT7-Luc. The cells were G418 selected and the colonies were quantified as described in panel (A). Results are normalised to the pLuc control, which is set at 100%. For (A) a single control bar set at 100% is graphed for simplicity. Values are the mean ± SD of 3 independent experiments.

in comparison to the non-silencing control in both HeLa and 293T cells. Similar experiments were performed with comparable results using a second, unrelated MOV10-specific shRNA (data not shown). To determine whether depletion of endogenous MOV10 affects multiple rounds of HIV-1 replication, we infected stable non-silencing control or MOV10 KD Hut78 T cells with equal amounts of HIV-$_{1NL4-3}$ and determined the effect on virus production. Consistent with the single-cycle infectivity assays, silencing of MOV10 had no effect on spreading HIV-1 replication (Figure 3C).

To determine whether endogenous MOV10 regulates the production of infectious SIVmac, MLV or M-PMV, virions were produced as described above in 293T non-silencing control or MOV10 KD cells and the effect on infectious particle production was determined. Depletion of endogenous MOV10 had no significant effect on the production of HIV-1, SIVmac, MLV or M-PMV infectious particles (Figure 3D). Although we cannot rule out the possibility that undetectable levels of residual MOV10 in our KD cultures are still functional, these data strongly suggest that endogenous levels of MOV10 do not control the replication of exogenous retroviruses.

Depletion of endogenous MOV10 specifically enhances the retrotransposition of endogenous retroelements

We next determined the effect of silencing endogenous MOV10 on LINE-1, Alu and IAP replication in the HeLa non-silencing control or MOV10 KD cells. In the absence of detectable MOV10, statistically significant 4-fold, 5-fold and 2-fold enhancements in retrotransposition frequencies were detected for LINE-1, Alu and IAP, respectively (Figure 4A). We also transfected HeLa non-silencing control or MOV10 KD cell lines with the pcDNA3.1-*neo* control plasmid and obtained similar number of colonies with the non- silencing control and MOV10 KD cell lines verifying that silencing of endogenous MOV10 does not effect *neo* expression and selection directly, and also has no effect on transfection efficiency (Figure 4B).

To confirm that the increase in endogenous retroelement replication was due to the depletion of endogenous MOV10 and not an unanticipated off-target effect, we constructed a silencing resistant MOV10 vector, pMOV10-R, by introducing silent mutations that prevented recognition by the shRNA. The antiviral activity of MOV10 was unaffected by these mutations as overexpression of pMOV10-R inhibited the production of infectious MLV and M-PMV

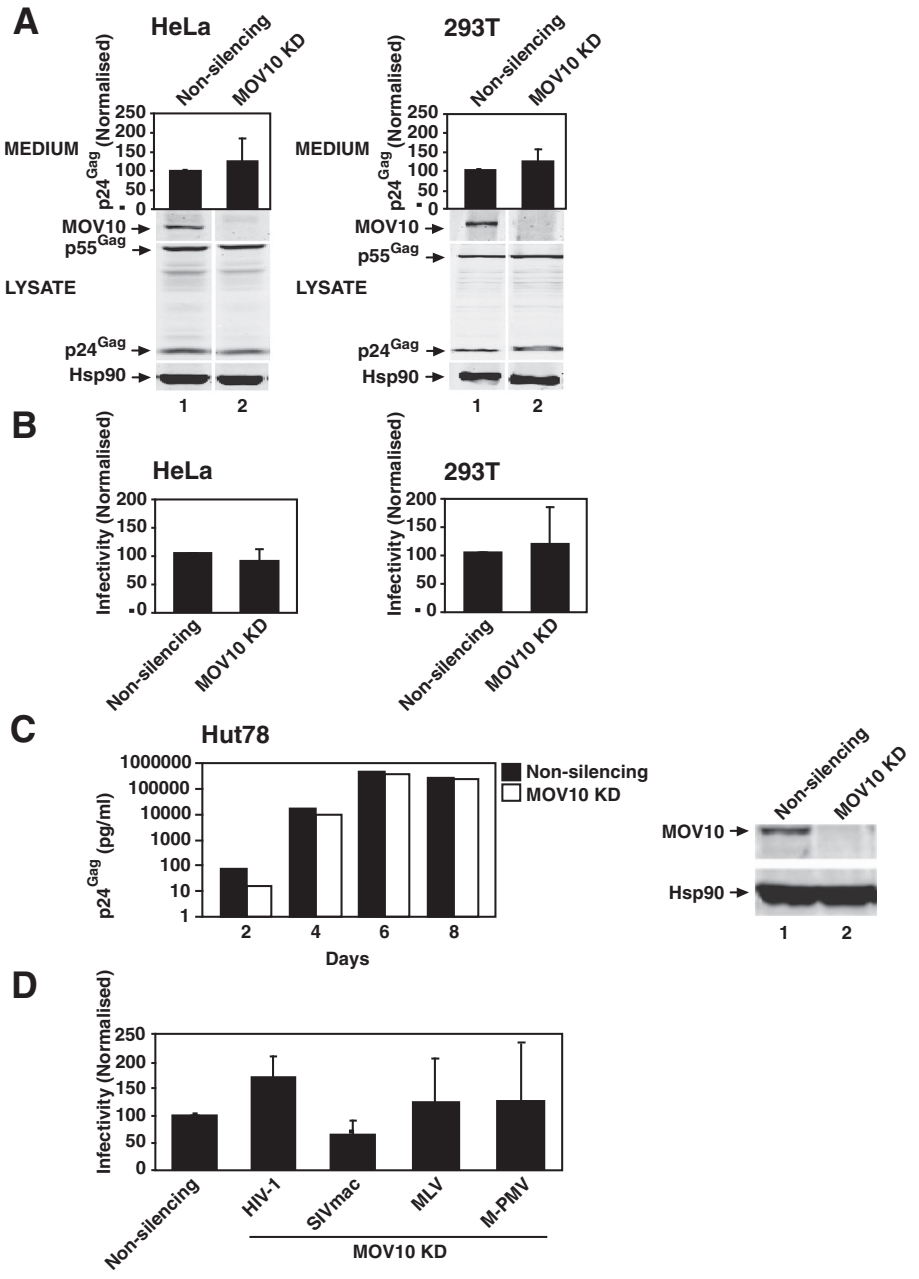

Figure 3 Silencing endogenous MOV10 has no significant effect on the production of infectious retrovirus particles for a panel of exogenous retroviruses. (A) Depletion of endogenous MOV10 has no effect on HIV-1 virus production. Stable MOV10 KD cells were produced by transducing HeLa or 293T cells with lentiviral vectors expressing either a non-silencing control shRNA or a MOV10-specific shRNA. HeLa or 293T non-silencing control and MOV10 KD cells were infected with VSV-G pseudotyped HIV-1_{NL4-3}. Virus concentration in the medium was determined as described in Figure 1A. Cell lysates were analysed by immunoblotting with anti-$p24^{Gag}$, anti-Hsp90 or anti-MOV10 antibodies, the latter of which was used to verify the MOV10 KD. (HeLa virus production p = 0.0611, 293T virus production p = 0.2007). **(B)** Silencing of endogenous MOV10 has no effect on HIV-1 virion infectivity. Virion infectivity was determined as described in Figure 1B. (HeLa infectivity p = 0.3080, 293T infectivity p = 0.4812). **(C)** Depleting endogenous MOV10 has no effect on spreading HIV-1 replication. Hut78 non-silencing control or MOV10 KD cells were infected with equal amounts of HIV-1_{NL4-3} and passaged every 2 days. Medium was harvested on days 2, 4, 6 and 8 and virus production was determined as described in Figure 1A. Cell lysates were analysed by immunoblotting with anti-MOV10 and anti-Hsp90 antibodies. **(D)** MOV10 silencing has no effect on the production of infectious SIVmac, MLV and M-PMV particles. 293T non-silencing control or MOV10 KD stable cells were transfected as described in Figure 1C for the production of HIV-1, SIVmac, MLV and M-PMV particles. Infectivity was determined as described in Figure 1C. (HIV-1 p = 0.1358, SIVmac p = 0.1040, MLV p = 0.4907, M- PMV p = 0.4919). For (A), (B) and (D) results are normalised to the non-silencing control, which is set at 100%. For (D) a single control bar set at 100% is graphed for simplicity. Values are the mean ± SD of 7 independent experiments for (A) and (B) or 3 independent experiments for (D). The data were analysed with an unpaired one- tailed *t* test.

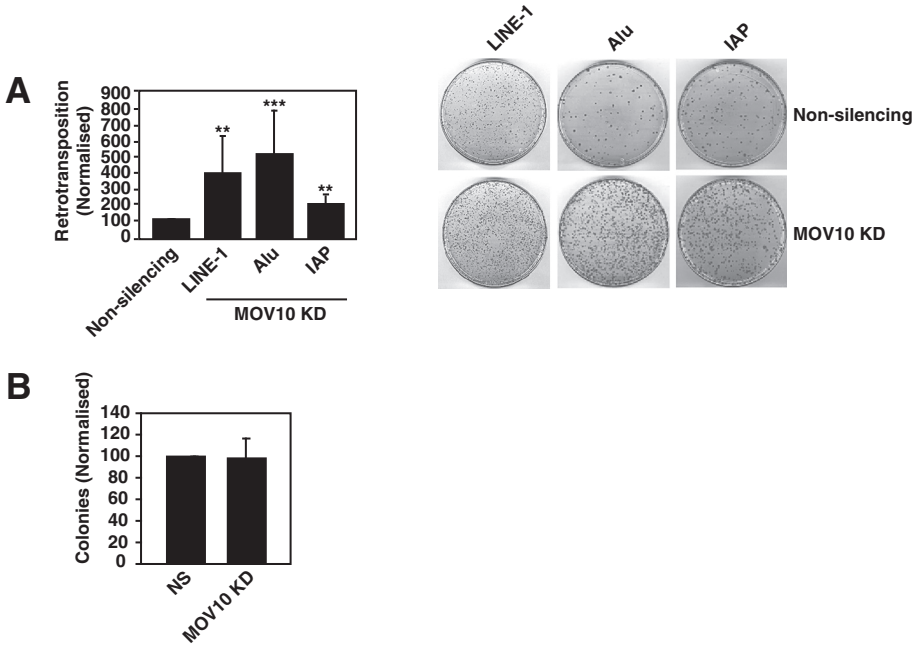

Figure 4 Depletion of endogenous MOV10 significantly enhances the retrotransposition of endogenous retroelements. (A) Silencing of endogenous MOV10 significantly enhances the retrotransposition of LINE-1, Alu and IAP. As described in Figure 2A, HeLa non-silencing control or MOV10 KD stable cell lines were transfected with retroelement expression plasmids and retrotransposition was quantified. (LINE-1 **$p \leq 0.0056$, Alu ***$p \leq 0.0005$, IAP **$p \leq 0.0096$). **(B)** MOV10 silencing does not effect *neo* expression or selection directly. As described in Figure 2B, HeLa non-silencing control or MOV10 KD cells were transfected with the pcDNA3.1-*neo* control vector and colonies were quantified. Results are normalised to the non-silencing control, which is set at 100%. For (A) a single control bar set at 100% is graphed for simplicity. Values are the mean ± SD of 3 independent experiments. The data were analysed with an unpaired one-tailed *t* test.

virions to a similar magnitude as parental pMOV10 (data not shown). The shRNA resistance of pMOV10-R was confirmed by titrating sensitive pMOV10 and resistant pMOV10-R into the HeLa MOV10 KD cells, and analysing cell lysates by immunoblotting (Additional file 1A). Results showed that the levels of MOV10 encoded by pMOV10-R were elevated in the KD cells relative to those seen with the parental pMOV10 vector (Additional file 1A, compare lanes 5 and 6 with 11 and 12). Next, we transfected MOV10 KD cells with pMOV10-R to test the functional consequence of restoring MOV10 expression, and found that the suppression of LINE-1 retrotransposition was re-established (Additional file 1B).

Thus, endogenous human MOV10 specifically represses the propagation of intracellular retroelements.

MOV10 is not necessary for miRNA or siRNA-mediated mRNA silencing

MOV10 interacts with the Argonaute proteins, which are central effector components of the RISC, and has been reported to be necessary for siRNA-mediated mRNA silencing by an endogenous miRNA [27]. To determine whether MOV10 is necessary for small RNA-mediated RNA silencing, which is one possible mechanism by which MOV10 may control the replication of endogenous retroelements, we initially tested the requirement of MOV10 for miRNA-mediated mRNA repression. HeLa non-silencing control or MOV10 KD cell lines were transfected with either a firefly (FF) luciferase reporter construct containing four copies of the endogenous let-7 miRNA binding site (FF4LCS; let-7 WT) or a negative control carrying mutations in the target seed region of the let-7 binding sites (FFr4mLCS; let-7 mutant), together with a control plasmid expressing renilla luciferase (pRenilla) [50]. Cells were lysed and the relative FF luciferase and renilla luciferase activities were determined. FF luciferase activity was normalised to the renilla luciferase activity to control for transfection efficiency.

As expected, the let-7 WT luciferase activity was repressed ~5-fold compared to the let-7 mutant luciferase activity in the non-silencing control cells (Figure 5A). A similar 5-fold repression in let-7 WT luciferase activity relative to the let-7 mutant luciferase activity was observed in the MOV10 KD cells suggesting that MOV10 is not required for endogenous let-7 miRNA-mediated mRNA repression in HeLa cells (Figure 5A). As a control for this assay, we also knocked down DICER-1, which is an RNase III enzyme essential for miRNA biogenesis, and co-transfected DICER-1 KD or non-silencing control cells

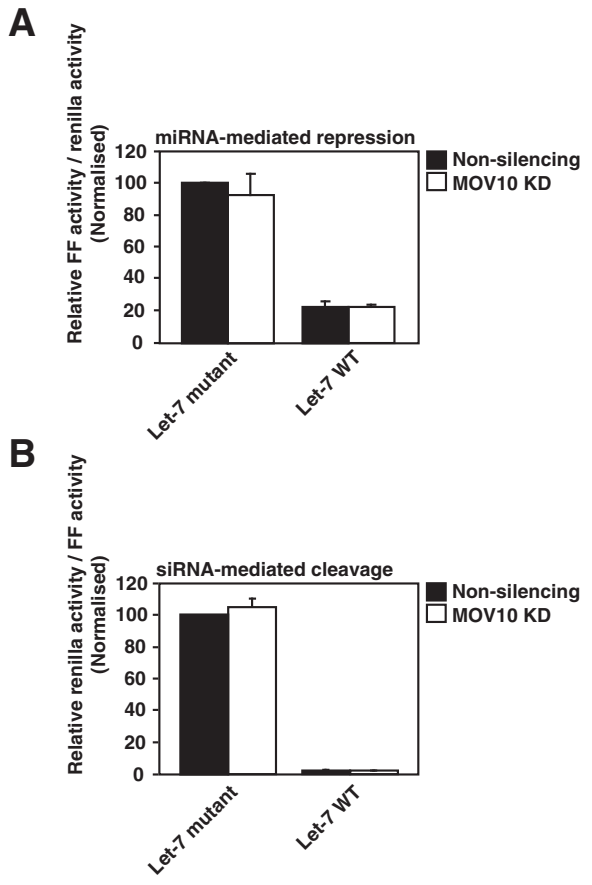

Figure 5 MOV10 is not necessary for miRNA or siRNA-mediated mRNA silencing. **(A)** MOV10 is not necessary for miRNA-mediated mRNA repression. HeLa non-silencing control or MOV10 KD cells were co-transfected with either FF4LCS (let-7 WT) or FFr4mLCS (let-7 mutant) together with pRenilla. Relative luciferase activities were measured using a Dual-Luciferase® Reporter Assay System and FF luciferase activity was normalised to the renilla luciferase activity. **(B)** MOV10 is not required for siRNA-mediated mRNA cleavage. HeLa non-silencing control or MOV10 KD cells were transfected with either psi-CHECK2-let-7X3 (let-7 WT) or psi-CHECK2-let-7X3m (let-7 mutant). Luciferase activities were measured as described in panel (A) and renilla luciferase activity was normalised to FF luciferase activity. Results are normalised to the non-silencing control, which is set at 100%. Values are the mean ± SD of 3 independent experiments.

with the FF luciferase reporter constructs and pRenilla. As expected, and confirming the validity of our approach, a 70% decrease in DICER-1 mRNA expression resulted in a partial derepression of the let-7 miRNA activity (Additional file 2).

To determine whether MOV10 is required for siRNA-mediated mRNA silencing as previously reported [27], HeLa non-silencing control or MOV10 KD cells were transfected with renilla luciferase reporter constructs containing three copies of perfectly complementary let-7 miRNA binding sites (psi-CHECK2-let-7X3; let-7 WT) or mutated let-7 binding sites (psi-CHECK2-let-7X3m; let-7 mutant) [51]. The perfect complementarity between endogenous let-7 miRNA and the reporter mRNA promotes siRNA-mediated cleavage instead of miRNA-mediated repression. Both let-7 WT and let-7 mutant constructs also expressed FF luciferase from a second promoter and the renilla luciferase activity was normalised to FF luciferase activity to control for transfection efficiency. In the non-silencing control cells, let-7 WT luciferase activity was repressed by ~50-fold relative to the let-7 mutant luciferase activity, and this level of repression was maintained in the MOV10 KD cells (Figure 5B). These results imply that, at least in HeLa cells, MOV10 is not essential for miRNA or siRNA-mediated mRNA silencing.

MOV10 is dispensable for the restriction of LINE-1 or HIV-1 replication by APOBEC3 proteins

Overexpression experiments have previously shown that a number of APOBEC3 proteins such as APOBEC3A (A3A), APOBEC3B (A3B) and A3G inhibit the retrotransposition of endogenous retroelements [6-8,10] and the depletion of endogenous A3B increases the replication of human LINE-1 in HeLa and human embryonic stem cells [10]. Since MOV10 was identified as an APOBEC3-interacting protein, we determined whether A3A, A3B or A3G require MOV10 for the restriction of LINE-1. HeLa non-silencing control or MOV10 KD cells were co-transfected with either pA3A, pA3B, pA3G or a pGFP control (pCMV4-HA tagged A3A, A3B, A3G or GFP) [52] together with pLINE-1 as described. A3A completely inhibited LINE-1 retrotransposition both in the presence or absence of MOV10 (Additional file 3A). A3B restricted LINE-1 replication in the non-silencing control and MOV10 KD cells by 70% and 66%, respectively, while A3G inhibited LINE-1 retrotransposition by 50% and 56%, respectively, relative to the non-silencing control and MOV10 KD pGFP controls (Additional file 3A). Therefore, A3A, A3B and A3G do not require MOV10 for the inhibition of LINE-1 mobilisation.

Similarly, we also tested whether MOV10 is required for A3G antiviral activity by co-transfecting HeLa non-silencing control and MOV10 KD cells with pA3G or a pGFP control together with a plasmid expressing a vif-deficient HIV-1 provirus (pHIV-1_{IIIB}/Δvif) [52]. Virion infectivity was determined using the TZM-bl reporter cell line and results showed that A3G still inhibited HIV-1 infectivity in the absence of endogenous MOV10, suggesting that MOV10 is not required for A3G antiviral activity (Additional file 3B).

Discussion

Host cell restriction factors inhibit the replication of a diverse range of exogenous retroviruses and endogenous

retroelements. Identifying the full complement of these proteins is necessary to understand the capacity of the host to regulate and control these genetic parasites. MOV10 has been reported to modulate the replication of a variety of RNA viruses including HCV, HDV and VSV [18-20]. Here, we analysed whether MOV10 controls the replication of exogenous retroviruses and endogenous retroelements.

Our results show that MOV10 overexpression restricts the production of infectious retrovirus particles (Figure 1). This broadly agrees with previously published reports [30-32], and extends the finding to the betaretrovirus M-PMV (Figure 1C and D). Similar to Furtak et al., [31] we observe a greater decrease in HIV-1 virion infectivity compared to virion production, and this is more obvious in 293T cells (Figure 1A and B). We also observe a modest decrease in cellular HIV-1 Gag abundance and processing similar to that reported by Burdick et al. [30], as well as a more noticeable decrease in cellular Gag abundance and processing for M-PMV (Figure 1A and D). As virion assembly is a cooperative process, decreases in total intracellular Gag abundance may account for the reductions in Gag processing [53]. MOV10 can also be packaged into budding HIV-1 virions [30,32-34] and, interestingly, the overexpression of MOV10 in HIV-1 producing cells decreases the accumulation of early reverse transcription products in target cells (data not shown) [31,32]. The mechanism(s) underlying the defects in virion production and reverse transcription are unclear, though the generality of these observations across retroviral genera suggests a common mode of action.

As described above, three groups have analysed the role of endogenous MOV10 in HIV-1 replication, but have reported variable results [30-32]. It was important for us to test the effect of depleting endogenous MOV10 on HIV-1 replication, and we extended this to include a panel of exogenous retroviruses. Contrary to the previous reports, we observe that depletion of endogenous MOV10 has no affect on the production of infectious retroviral particles or spreading HIV-1 replication (Figure 3). This result is similar to that reported recently for foamy virus, a distantly related retrovirus belonging to the spumaretrovirus subfamily, where knockdown of MOV10 had no effect on viral replication [54]. In sum, while it appears that endogenous levels of MOV10 do not restrict retroviral replication, we speculate that the results of overexpression studies implicate MOV10 as a component of a pathway or multiple pathways that exogenous retroviruses encounter. MOV10 has also been reported to be a type I interferon-stimulated gene [19], but whether interferon or other cytokines can stimulate sufficient levels of MOV10 protein to impact exogenous retrovirus infections is not yet known.

The MOV10 ortholog Armitage is required for the repression of endogenous mobile elements in both germ cells and somatic cells in *Drosophila melanogaster* [22,25,26]. Similarly, the MOV10 paralog, MOV10L1, has been shown to be necessary for the silencing of endogenous retrotransposons in the germ line of male mice [23,24]. Therefore, we analysed whether human MOV10 could inhibit endogenous retroelements. Similar to its effect on exogenous retroviruses, the overexpression of MOV10 potently inhibits the transposition of the human endogenous retrotransposons LINE-1 and Alu as well as the mouse endogenous retrovirus IAP (Figure 2A). Unlike the exogenous retroviruses, however, the depletion of endogenous MOV10 significantly enhances the replication of LINE-1, Alu and IAP (Figure 4A), which in the case of LINE-1 can be reversed by restoration of MOV10 expression with an shRNA-resistant version of MOV10 (Additional file 1). The mechanism by which MOV10 controls these LTR and non-LTR endogenous retroelements is unknown, but previous studies have shown that *Dicer1* knockout mouse embryonic stem cells have increased levels of LINE-1 and IAP transcripts [55]. Furthermore, Yang et al., [56] showed that human LINE-1 bidirectional transcripts produced from the LINE-1 sense and antisense promoters (ASP) are processed to yield LINE-1 specific endogenous siRNAs that suppress LINE-1 retrotransposition by an RNAi mechanism.

Although it has been reported that MOV10 associates with the RISC pathway and is necessary for siRNA-mediated silencing of target mRNAs [27], our findings to date using reporter constructs indicate that MOV10 is not absolutely required for miRNA or siRNA-mediated mRNA silencing in cultured cells (Figure 5A and B); therefore, whether this is a mechanism by which MOV10 could regulate endogenous retroelements is unclear. MOV10 also localises to mRNA processing bodies (PBs) [27,29], which are cytoplasmic sites involved in the storage and decay of translationally repressed RNA species, and it has recently been reported that silencing of the PB-associated proteins DDX6 and 4E-T increases IAP transcript levels and promotes IAP retrotransposition [57]. Taking this into consideration, we are currently investigating the pathway/mechanism by which MOV10 regulates retroelement mobility.

Human MOV10 is expressed in a wide range of adult tissues including the heart, lungs, liver, testes and ovaries with the highest transcript levels detected in the adult brain including the hippocampus and caudate nucleus [58]. Intriguingly, recent studies have shown that LINE-1 transcripts are expressed in most human somatic tissues as opposed to being confined to the germ line [3]. Furthermore, active LINE-1, Alu and SVA element retrotranspositions in the human hippocampus and caudate nucleus have been reported to contribute to the

genetic mosaicism of the human brain that may underlie both normal and abnormal neurobiological processes [2,4]. Based on our observations that endogenous MOV10 regulates LINE-1 and Alu replication (Figure 4A), it will be interesting to determine whether human MOV10 may be involved in the modulation of somatic retrotransposition and contribute to the control of retrotransposition-mediated genetic variation.

Conclusion

MOV10 overexpression potently restricts the replication of a broad range of exogenous and endogenous retroelements. Silencing endogenous MOV10 has no effect on the replication of exogenous retroviruses, but it significantly enhances the retrotransposition of endogenous retroelements. We hypothesise that MOV10 may contribute to the regulation of endogenous retroelement mobilisation in somatic cells.

Methods

Cell culture, MOV10 RNAi and plasmids

Human HeLa and 293T cells were cultured in Dulbecco's modified Eagle's medium while Hut78 cells were cultured in RPMI. Both types of media were supplemented with 10% fetal bovine serum plus penicillin-streptomycin and L-glutamine. 293T cells were co-transfected with lentiviral vectors expressing either a non-silencing control or MOV10-specific shRNAmir in the miR-30 context containing a puromycin resistance gene (GIPZ Lentiviral shRNAmir, Open Biosystems V2LHS_201304), together with the HIV-1 p8.91 packaging plasmid and pVSV-G (see plasmids below). HeLa, 293T and Hut78 cells were transduced with the recombinant lentiviral stocks and stably transduced cells were selected with puromycin treatment.

The pT7-MOV10 and pT7-Luc plasmids were constructed by cloning XbaI-BamHI digested full-length MOV10 and FF luciferase PCR products into the $pCGTHCF_{FL}T7$ expression vector that contains two 5′-T7-epitope tags [59]. The pMOV10-R plasmid was constructed by introducing six silent mutations into the MOV10-specific shRNA target sequence (nucleotides 342 to 363) by overlapping PCR (Primers: Forward 5′ TTTATGACAGGGC***C***GA***A***TA***C***CT***C***CA***C***GG***A***AAA CATGGTGTGG 3′, Reverse 5′ CCACACCATGTTT ***T***CC***G***TG***G***AG***G***TA***T***TC***G***GCCCTGTCATAAA 3′) and cloning the XbaI-XmaI digested PCR product into a similarly digested pT7-MOV10 vector. The $HIV\text{-}1_{NL4\text{-}3}$ strain provirus was used for this study [35]. Plasmids for exogenous retrovirus and endogenous retroelement experiments have been described previously: pVSV-G [37]; HIV-1, p8.91 and pCSGW [38,39]; SIVmac, pSIV3+ and pSIV-RMES4 [36]; MLV, pNCS/FLAG and pMSCV/Tat [40,41] M-PMV, pMTΔE [42]; LINE-1, pJM101/L1.3 [47]; Alu, pAlu-neo^{Tet} and pCEP-ORF2 [48]; IAP, pGL3- IAP92L23neo^{TNF} [49]. Plasmids for the luciferase assays were described previously: FF4LCS, FFr4mLCS, pRenilla, psi-CHECK2-let-7X3 and psi-CHECK2-let-7X3m [50,51].

Virus production and infectivity assays

For wild-type HIV-1 virus production, parental HeLa or 293T cells (2 x 10^5 cells) were transfected with 0.5 μg of a plasmid expressing the full-length $HIV\text{-}1_{NL4\text{-}3}$ strain provirus ($pHIV\text{-}1_{NL4\text{-}3}$) using either FuGENE 6 (Roche) according to manufacturer's instructions at a 3 μl FuGENE to 1 μg DNA ratio for the HeLa cells, or 16 μl (1 mg/ml) PEI (per well of a 6-well dish) for the 293T cells. For MOV10 overexpression experiments, $pHIV\text{-}1_{NL4\text{-}3}$ was co-transfected with the indicated concentration of pT7-MOV10 and the appropriate concentration of the pT7-Luc control plasmid to ensure equivalent amounts of DNA in all transfections. For HIV-1 and SIVmac lentiviral vector production, parental 293T cells or 293T non-silencing control and MOV10 KD cells were co-transfected as described with 1 μg p8.91, 1 μg pCSGW and 0.5 μg pVSV-G, or 1 μg pSIV3+, 1 μg pSIV-RMES4 and 0.5 μg pVSV-G, respectively. MLV and M-PMV virions were produced by co-transfecting parental 293T cells or non-silencing control and MOV10 KD cells as described with 0.2 μg pNCS/FLAG, 0.2 μg pMSCV/Tat and 0.1 μg pVSV-G, or 1 μg pMTΔE and 0.5 μg pVSV-G, respectively. Plasmids for lentiviral vector or MLV and M-PMV virion production were co-transfected with 0.5 μg pT7-MOV10 or pT7-Luc for MOV10 overexpression experiments. Cells were lysed ~40 h post-transfection and virus particles were filtered through a 0.45 μM filter. The concentration of HIV-1 $p24^{Gag}$ in the supernatant was quantified by a $p24^{Gag}$ enzyme-linked immunosorbent assay (ELISA) (Perkin-Elmer).

For HIV-1 infectivity, the TZM-bl reporter cell line (1 x 10^5 cells) expressing a HIV-1 Tat inducible β-gal reporter gene was challenged with equal amounts of virus normalised by the $p24^{Gag}$ concentration. Cells were lysed ~24 h post-infection and β-gal activity was determined using the Galacto-Star™ System (Applied Biosystems) according to the manufacturer's instructions. For HIV-1 and SIVmac lentiviral vector infectivity, 293T cells (1 x 10^5 cells) were challenged with equal amounts of vector- containing medium and infectivity was determined ~24 h post-infection by measuring the percentage of GFP-positive 293T cells using a FACS Canto II Flow Cytometry System (BD Biosciences). MLV and M-PMV virion infectivity was determined by infecting TZM-bl cells (1 x 10^5 cells) with equal amounts of virus-containing medium and infectivity was determined as described for the wild-type HIV-1 experiments.

HIV-1 infection of producer cells and spreading infection

For endogenous MOV10 silencing experiments, HeLa or 293T non-silencing control and MOV10 KD cells (2 x 10^5 cells) were infected with equal amounts of VSV-G pseudotyped wild type HIV-1_{NL4-3} virus normalised by the $p24^{Gag}$ concentration (25 ngs) in a total of 1 ml medium (6-well dish). The cells were washed 4 h later and 2 mls of fresh medium was replaced. For spreading replication, non-silencing control or MOV10 KD Hut78 cells (1 x 10^6 cells) were infected with equal amounts of virus normalised by the $p24^{Gag}$ concentration (100 ngs) and cells were passaged every 2 days. Medium was harvested on days 2, 4, 6 and 8 and virus production was measured by $p24^{Gag}$ ELISA.

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) and filtered virions were pelleted through a 20% sucrose cushion and lysed. Proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting. HIV-1 precursor $p55^{Gag}$ and processed $p24^{Gag}$ were detected using a mouse anti-$p24^{Gag}$ antibody [60]. M-PMV precursor $p78^{Gag}$ and processed $p27^{Gag}$ were detected using goat anti-$p27^{Gag}$ antisera (78 S-136, Microbiological Association). T7-tagged MOV10 and Hsp90 were detected with mouse anti-T7 (Novagen) and rabbit anti-Hsp90 (Santa Cruz Biotechnology) antibodies, respectively. Endogenous MOV10 was detected with a rabbit anti-MOV10 antibody (Proteintech). Secondary IRdye800 conjugated antibodies (Li-Cor Biosciences) were used for quantitative immunoblotting with the Odyssey infrared scanner (Li-Cor Biosciences).

Retrotransposition assays

For LINE-1, Alu and IAP retrotransposition assays parental HeLa cells or HeLa non-silencing control and MOV10 KD cells (2 x 10^5 cells) were co-transfected as described with either 1.5 μg pJM101/L1.3, 1 μg pAlu-neo^{Tet} plus 0.5 μg pCEP-ORF2 or 1.5 μg pGL3-IAP92L23neo^{TNF}, respectively. Plasmids were co-transfected with 1 μg pT7-MOV10 or pT7-Luc for MOV10 overexpression experiments. Cells were G418 selected (1 mg/ml) 2 days post-transfection. At ~12-15 days post-transfection, the cells were fixed in 4% paraformaldehyde and colonies were stained with 0.4% Giemsa (Sigma) for counting. For control pcDNA3.1-*neo* experiments cells were transfected with 0.3 μg of a pcDNA3.1 empty vector containing a neomycin resistance cassette, and the assay was performed similarly to the retrotransposition assays.

Luciferase assays

For the miRNA assays, HeLa non-silencing control or MOV10 KD cells (1 x 10^5 cells) were co-transfected as described with either 0.1 μg FF4LCS or FFr4mLCS together with 0.1 μg pRenilla. For the siRNA assays, the cells were transfected with 0.1 μg psi-CHECK2-let-7X3 or psi-CHECK2-let-7X3m. Cells were lysed ~24 h post-transfection. Relative luciferase activities were measured using a Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions.

Additional files

Additional file 1: Restoration of MOV10 expression rescues the control of LINE-1 retrotransposition. (A) HeLa MOV10 KD cells were transfected with increasing concentrations of >pT7-MOV10 or pT7-MOV10-R. Cells were analysed by immunoblotting with anti-MOV10, anti-T7 and anti-Hsp90 antibodies. **(B)** HeLa non-silencing control or MOV10 KD cells were co-transfected with pLINE-1 (pJM101/L1.3) together with pT7-MOV10-R or pT7-Luc at the indicated concentrations, following which the cultures were G418 selected and colonies were counted to measure the retrotransposition frequency. Cell lysates were analysed by immunoblotting with anti-MOV10, anti-T7 and anti-Hsp90 antibodies. For (B) results are normalised to the non-silencing control, which is set at 100%. Values are the mean ± SD of 3 independent experiments.

Additional file 2: Knockdown of DICER-1 relieves miRNA-mediated mRNA repression. HeLa cells were transfected with non-silencing control or DICER-1-specific siRNAs to produce non-silencing control or DICER-1 KD cells, respectively. These cells were co-transfected with either FF4LCS (let-7 WT) or FFr4mLCS (let-7 mutant) together with pRenilla. The relative luciferase activities were measured using a Dual-Luciferase® Reporter Assay System. FF luciferase activity was normalised to renilla luciferase activity.

Additional file 3: MOV10 is not required for restriction of LINE-1 or HIV-1 infection by APOBEC3 proteins. (A) HeLa non-silencing control or MOV10 KD cells were co-transfected with pLINE-1 (pJM101/L1.3) and pCMV4-HA tagged A3A, A3B, A3G or a GFP control. Cells were G418 selected and colonies were quantified to determine the retrotransposition frequency. **(B)** HeLa non-silencing control or MOV10 KD cells were co-transfected with pHIV-1_{IIIB}/Δvif and either pA3G or pGFP. Infectivity was determined by infecting TZM-bl cells with equal amounts of virus normalised by the $p24^{Gag}$ concentration. For (A) results are normalised to the non-silencing control, which is set at 100%. Values are the mean ± SD of 3 independent experiments.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SA conducted the experiments, performed the analyses and interpretation of the data, and wrote the manuscript. CMS and MHM helped write the manuscript. CMS, NMS and MHM conceived the study and contributed to data interpretation, and MHM supervised the project. SMW provided experimental tools for the study. NMS and SMW also contributed to the drafting of the manuscript. All authors read and approved the final manuscript.

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